Manual of methods in aquatic environment research

Part 10 - Short-term static bioassays

by
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PREPARATION OF THIS DOCUMENT

At its Seventh Session in October 1973, the FAO Advisory Committee on Marine Resources Research (ACMRR) agreed to establish, jointly with the International Association for Biological Oceanography (IABO), a Working Party on Biological Effects of Pollutants, whose task would be to review and evaluate critically the methods for bioassay and toxicity tests presently used and, specifically, to advise on the practical use of the tests and to identify research programmes required to improve methodology and their application.

Under the cooperative project of the United Nations Environment Programme (UNEP) entitled, "Effects of Pollutants on Aquatic Resouces and Scientific Basis for Monitoring", the Working Party held two sessions and produced as its end product the "Manual of Methods in Aquatic Environment Research, Part 4, Basis for Selecting Biological Tests to Evaluate Marine Pollution" (FAO Fish. Tech. Pap. (164), 1977). The senior author was a member of this committee and attended the second meeting in Dubrovnik, Yugoslavia, on 22-25 November 1976. The Working Party recommended that manuals be prepared to assist laboratories in developing countries. The first of these manuals was entitled, "Manual of Methods in Aquatic Environment Research, Part 6 - Toxicity Test" FAO Fish. Tech. Pap. (185), 1982. The present manual (number 10) is the second one and has been prepared by D. J. Reish and P. S. Oshida, Department of Biology, California State University, Long Beach, CA, U.S.A. 90840. Final editing was done by H. Naeve, Fishery Resources and Environment Division, FAO. The reader is directed also to the manual entitled, "Manual of Methods in Aquatic Environment Research, Part 7 - Selected Bioassays for the Mediterranean" (FAO Fish Tech. Pap. (208), 1981).

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The views expressed in the manual are those of the authors and do not necessarily represent the views of FAO.

DEFINITION OF MARINE POLLUTION

Pollution of the marine environment means: "The introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) which results in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of sea water and reduction of amenities".

IMO/FAO/Unesco/WMO/WHO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP)

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SUMMARY

The data obtained from bioassays are useful in assessing the effects of toxicants in the aquatic environment. Bioassays utilize different measures of toxicity for various lengths of exposure, to determine whether a particular substance is harmful to the environment. This manual was designed for laboratories with limited facilities, equipment, and supplies. All tests described herein are static and of short-term duration (96 hours). They measure the acute toxicity of a single or complex solution of pollutants. The manual describes the preparation and procedures for tests with fresh water and marine phytoplankton, annelids, zooplankton, crustaceans, aquatic insects, molluscs, echinoderm larvae, and fish. The different procedures utilized in data analysis are described in detail and examples are given. Bioassay procedures are given using selected toxicants.

Cover photo: Donald J. Reish setting up a static bioassay with the polychaete Neanthes arenaceodentata. Photograph by Robert Freligh, Audio-Visual Center, California State University, Long Beach, USA

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1. INTRODUCTION

1.1 Purposes of Bioassays

Bioassays, or toxicity tests, are conducted to measure the effect of one or more pollutants on one or more species of organisms. Tests are conducted for various lengths of time, but the 96 hour test is the most common and the one which will be described in this manual. Test organisms are exposed to increasing concentrations of a toxicant in order to determine some change in the organism. Death is generally used as a criteria of a change in the 96 hour test. One or more controls are employed in which the organism is exposed to similar conditions except lacking the toxicant to provide a measure of experimental acceptability. The concentration which causes a 50% live-death response is defined as the lethal concentration, or LC50. These experiments are conducted under controlled laboratory conditions. The data generated, in the form of 96 hour LC50s, are used to evaluate the effect of a toxicant being discharged into the aquatic environment. These data assist in the development and application of water quality criteria for protection of the aquatic environment.

1.2 Types of Bioassays

The term 'bioassay' is used in this manual according to the definition given on page 5 in FAO (1977) which states:

> "Bioassay signifies a test in which a living tissue, organism or group of organisms is used as a reagent for the determination of the potency of any physiologically active substance of unknown activity."

'Bioassay' has different meanings in different countries. Some workers prefer the term 'toxicity test' rather than bioassay since the experiment, or test, is actually measuring the toxic effect of a chemical or substance on living organisms. Others use these terms interchangeably. The term 'bioassay' will be used in this manual.

Many different types of bioassays have been utilized in the past from the simpler short-term test to highly sophisticated long-term experiments. In addition, the individual researcher has modified a particular test procedure to fit his needs or interests. Therefore, it is important in comparing results that the same experimental protocol was followed. Results can not be compared if one or more of the parameters, such as temperature, length of experiment, species of organism, etc., differ in the two experiments. Efforts towards the standardization of bioassay procedures have been made by various organizations such as the American Public Health Association (APHA/AWWA/WPCF, 1985), The American Society for Testing and Materials (Buikema and Cairns, 1980), the Food and Agriculture Organization of the United Nations (FAO, 1977; Bellan, 1981; Ward and Parrish (1981) and by workshops (Beynon and Cowell, 1974).

Bioassays are defined according to the duration of the experiment and the methods of adding the toxicant to the test solution. These tests are almost exclusively carried out under controlled laboratory conditions; however, in recent years field toxicity experiments have been conducted. While the emphasis herein will be on the 96 hour LC50 static bioassay, other types of tests, which require more time, equipment, and facilities, are described and documented for possible future use.

1.2.1 Short-term tests

This type of bioassay is conducted for a short period of time, usually 48 or 96 hours. The 96 hour test period is the most frequently used time interval; fish bioassays are occasionally run for a 48 hour period because of technical difficulties with some species. Organisms, except phytoplankton, echinoderm larvae, and possibly zooplankton, are not fed during the test and the solution is not changed. Death of the test organism is the criterion used to evaluate the toxicity of the solution. The 96 hour bioassay is advantageous because of minimal laboratory requirements and rapid results.

1.2.2 Long-term tests

This type of bioassay is conducted from 7 days to one or more months depending upon the species used and the type of data desired. Some long-term tests are simply extensions of the 96-hour test period which generally involve feeding the organisms and may involve renewing the test solution. Death is also used as the criterion for toxicity in this type of long-term test. Other long-term tests involve growth or reproduction as a measure of sublethal toxicity. More time and experience is required in order to conduct this type of experiment. The advantage of these long-term tests is they indicate more realistically whether or not a particular species can live at a particular concentration. Results from each test concentration are compared to the control. Criteria for comparisons include growth rate, whether or not reproduction occurs, and the number of eggs laid or larvae produced.

1.2.3 Static tests

This type of bioassay is generally of short duration in which the organism is kept in the same test solution for the entire experimental period. The 96 hour bioassay is almost always of the static type.

1.2.4 Renewal tests

Bioassays in which the test solution in each experimental container is changed periodically with fresh solution. The renewal period varies according to the species of organism and the toxicant. This type of bioassay requires some additional time for changing the test solutions.

1.2.5 Flow-through tests

A highly sophisticated type of bioassay in which the test solution is renewed continuously. This type of test is perhaps more realistic of field conditions, but there are many inherent problems connected with this type of test, such as large space requirements, large quantities of water used, and a complex water delivery system. Flow through bioassays are not recommended for personnel who have had little or no previous experience conducting bioassays or with limited laboratory facilities and budget.

1.2.6 Other types of bioassays

Many other types of bioassays have been conducted which are dictated by unique, local circumstances or the interest of the experimentor. The tests described below have not been utilized with the frequency of the above defined tests. They are listed for the edification of the reader to give him a broader understanding of the field of bioassay research.

1.2.6.1 Comparative toxicity

Results from bioassays are frequently compared with other data in two ways: comparison of the toxicity of many different toxicants to the same species of test organism and comparison of two or more test organisms to the same toxicant. Generally, but not necessarily, comparative toxicity tests are conducted for a 96 hour period. Comparative toxicity tests are valuable in giving the researcher greater understanding in interpreting the results. These tests, on the one hand, indicate which element or compound is the most toxic and thus assist the investigator in potential point source control of that waste, and, on the other hand, they will indicate which test organism is the most sensitive which will be useful in planning future bioassays. Whenever possible, more than one species of test organism should be used in separate 96 hour LC50 bioassays for testing the same toxicant; by following this practice, all routine bioassays are, in fact, comparative toxicity tests.

1.2.6.2 Biostimulation

This type of test procedure is used to evaluate the effect of a toxicant on primary productivity. Unicellular algae are taken from a laboratory stock culture and are grown for a specified period of time under standard conditions. Biostimulation is measured either by the amount of dry weight produced or by the number of cells per unit volume. Results are compared to control. Toxicants often consist of a mixture such as sewage effluent or other organic wastes. Replication is necessary because of variability of results from the same test concentrations in order to obtain statistically reliable results.

1.2.6.3 Growth rate

This type of test is useful as a sublethal measurement; however, it requires additional time, feeding, and either a flow-through system or periodically renewing the toxicant. Growth rate can be quantified by either determining the body weight or by measuring the length of the test organism. These measurements should be performed periodically during the course of a long-term experiment; extreme care must be exercised to avoid injury to the test organism while taking measurements. Specimens should be measured individually so that means and standard deviations can be calculated for the organisms in each concentration.

1.2.6.4 Bioaccumulation

In this type of test the researcher measures the amount of toxicant which has accumulated in the organism during the period of the experiment. Hydrocarbons, pesticides and heavy metals are the most commonly analyzed toxicants. Equipment needs to conduct this type of experiment are extensive and expensive. An atomic absorption spectrophotometer and gas chromatographic instrument plus supplies and an analytical laboratory are required in order to conduct these analyses. This type of test is frequently conducted in connection with other bioassays. Organisms, which have survived the test period, are analyzed for the presence and concentration of the toxicant. A minimum depuration period of 24 hours is required at the end of the test if the organims have been fed during the course of the experiment. The entire body is analyzed if the test organism is small; separate organs, such as liver, kidney, muscle, brain, and digestive system can be analyzed individually in larger organisms such as fish. Sufficient numbers of analyses must be made in order to obtain statistically reliable data.

1.2.6.5 Community response tests

This type of bioassay attempts to obtain a clearer idea of what happens in the field. Various experimental conditions are designed. For example, large compartmentalized containers, each one containing varying degrees of contaminated sediment, are placed in the field. After a period of time, at least a month or so, the population in each container is analyzed as to species composition, number of specimens, and weight. A minimum of three replicates is required for each environmental condition, and the data from each set are compared to the others. Another approach to the community response bioassay is to use the same experimental set-up but instead of placing them in the field they are kept in the laboratory where unfiltered sea water is allowed to drip on each compartment separately. Larvae from the ocean will come in via the sea water system and settle in the substrate. The population which develops in the compartment is compared to the others and related to the degree of contamination of substrate. A continuous dripping of the toxicant into each compartment may be required. A minimum of three replicates is required for this test. The community response test is a unique approach attempting to relate bioassay data to actual controlled field conditions. This test requires a seaside location as well as thorough knowledge of the fauna.

2. GENERAL BIOASSAY PROCEDURES

2.1 Test Organisms

2.1.1 Selection of test organisms

The selection of the test organism for the 96 hour bioassay is dependent upon many criteria which can be grouped according to the organism, the laboratory facilities and the researcher. The choice of species to be used should represent different habitats (i.e. pelagic, epibenthic, benthic), different trophic levels (i.e. primary producer, herbivore, carnivore, detrital feeder, etc.), and from different groups (i.e. phytoplankton, annelids, molluscs, arthropods, fish, etc.).

2.1.1.1 Laboratory facilities

Careful consideration must be given to the facilities, equipment, supplies, and needs of the laboratory in order for the bioassay to be conducted in a scientific and meaningful way. An adequate source of a good quality of fresh and/or marine water is required which is free of contaminants. Since toxicity may vary with temperature, it is essential that some method of maintaining constant temperature conditions be available where the bioassays are conducted.

2.1.1.2 Researcher

Laboratory personnel conducting the bioassays should have had previous experience working with the test organism. It is important that he have some previous knowledge of collecting and handling the organisms so that he will be able to distinguish normal and abnormal behaviour as well as living and dead specimens. If no one has had previous experience with the particular species, preliminary bioassay experiments should be conducted to gain background knowledge of the test organisms and procedures.

2.1.1.3 Organism

Many criteria need to be considered in the selection of the test organism; these include: availability of the species, ability of the species to live in the laboratory, a convenient size for handling and keeping under laboratory conditions, a previous knowledge of the species' biology including behaviour, feeding habits, ecology, reproduction, development, and life cycle, widely distributed over the area of concern, ecologically significant, and of economic value. It is unlikely that one species will satisfy all these criteria but they will serve as useful guidelines in making selections.

In the report, "Bases for selecting biological tests to evaluate marine pollution" (FAO, 1977), the following groups of organisms were recommended (some additional comments have been added):

Phytoplankton and zooplankton - they are convenient because of their small size, require a small amount of laboratory space, nutritional requirements often known, short life cycle, and good for bioaccumulation studies. The disadvantages include their small size which requires microscopic and specialized equipment for identification and counting.

Annelids including oligochaetes (fresh water) and polychaetes (salt water) - the advantages of this group are similar to those listed for phytoplankton and zooplankton. Their larger size allows for easier analysis.

Crustaceans - larvae from barnacles and decapods have been used in many bioassays. Large numbers can be otained from the mantle cavity of barnacles and the abdominal region of decapods. Adult isopods, amphipods, and decapods are useful because of their sensitivity to synthetic organic compounds. Crustaceans are especially sensitive during the molting stage. Species must be isolated in the test chamber because of their fighting and cannibalistic tendencies.

Molluscs - pelecypods, especially oysters and mussels, have been widely used as test organisms. They are easy to collect, handle, and maintain. Growth of test bivalves can easily be determined by the periodic measurement of the shell lengths in long-term tests. They are good bioaccumulators. Specimens produce large numbers of eggs and larvae which can be used in the same way as those from echinoderms. The disadvantages of pelecypods in 96 hour tests is the difficulty of determining death. Large quantities of water and phytoplankton as food are required to maintain bivalves in long-term tests.

Echinoderms - the eggs and larvae from sea urchins are useful because the adults are easily collected and large numbers of eggs and larvae can be obtained. The disadvantage is that the reproductive season may be of short duration especially in the more temperate climates.

Fish - are a major test organism because of their economic importance. Their biology is well known and they are usually sensitive. At least one species of fish should be included with every series of bioassay tests. The disadvantages are the difficulty in collection, handling, and transporting them to the laboratory. Larger laboratory facilities are required for the acclimation period as well as for the test period.

2.1.2 Collection of test organisms

While some species, such as algae (phytoplankton), zooplankton, and polychaetes have been successfully cultured through many generations in the laboratory, the source of most test organisms is from the natural environment. Collection of healthy organisms includes careful collection in the field, transportation to the laboratory which may include aeration and temperature control depending upon the distance involved, and adequate holding facilities. Collection techniques vary according to the type of organism, the facilities available, distance from the collecting site, and the tides (for marine organisms). Because of these variations, each of the groups listed above will be described separately.

2.1.2.1 Phytoplankton

Fresh water and marine phytoplankton can be collected from the field using a fine mesh plankton net. However, this will involve a considerable amount of time since specimens will have to be isolated and cultures established. It is much more convenient to obtain an established culture from one of several laboratories located in Europe and the United States. Phytoplankton is routinely transported by air. Many of the laboratories also furnish specific directions for culturing the particular species. Some laboratories where cultures can be obtained are:

Denmark:
Water Quality Institute
11 Agern Alle
2970 Hørsholm

France:
Station Marine d'Endoume
Rue de la Batterie des Lions
13007 Marseille

Germany, Federal Republic of:
Collection of Algae Cultures
Institute of Plant Physiology
University of Göttingen
Nikolausberger Weg 18
D-3400 Göttingen

Netherlands:
TNO Netherlands Organization for Applied Scientific Research
P.O. Box 217
2600 AE Delft

United Kingdom:
Marine Biological Association
The Hoe
Plymouth, England

Culture Centre of Algae and Protozoa
36 Storey's Way
Cambridge CB3 ODT
England

United States of America:
Algae Culture Collection
Dept. of Botany
University of Texas
Austin, Texas 78712

Scripps Institute of Oceanography
University of California
San Diego, California 92037

Woods Hole Oceanographic Institute
Woods Hole, Mass. 02543

Contact the supplier prior to the request for living material for supplies and equipment necessary to culture the species desired. The following are some of the more commonly used algae in bioassays.

Fresh water:
Chlorophyta (green algae)
Selenastrum capricornutum
Cyanophyta(blue-green algae)
Anabaena flos-aquae
Microcystis aeruginosa
Chrysophyta (diatoms)
Cyclotella sp.
Nitzschia sp.
Synedra sp.
Marine:
Chlorophyta (green algae)
Dunaliella tertiolecta
Chrysophyta (diatoms)
Skeletonema costatum
Thalassiosira pseudonana

2.1.2.2 Zooplankton

Fresh water and marine crustaceans can be collected with a fine mesh plankton net; however, because this method of collection concentrates the organisms, it will be necessary to provide aeration during transportation to the laboratory if the distance is greater than a few kilometers. Often, one species of crustacean will dominate the population of plankton. Care must be exercised to ensure that all specimens belong to the same species. Remove healthy appearing specimens with a pipette and place in a separate container for the holding period. The fresh water cladoceran *Daphnia* can be purchased from many biological supply companies. Members of the harpacticoid copepod genera *Tisbe* and *Tigriopus*, while not necessarily always inhabitants of the pelagic environment, have been used as test organisms. *Tisbe* can be collected from the surface of algae and *Tigriopus* from tide pools. Different species of the crustacean group Mysidacea have been used in bioassays. They are collected with either plankton nets or dip nets from the water surrounding offshore kelp beds. The culture methods for one species, *Mysidopsis bahia*, have been worked out. This has resulted in its use in many bioassays. The following are some of the more commonly used zooplankton in bioassays:

Fresh water:
- Cladocera
  - Daphnia magna

Marine:
- Copepoda
  - Acartia tonsa
  - Tisbe sp.
  - Tigriopus sp.
- Mysidacea
  - Acanthomysis sp.
  - Metamysidopsis sp.
  - Mysidopsis bahia

2.1.2.3 Annelids

Both oligochaetes, which are largely fresh water inhabitants, and polychaetes, which inhabit marine waters, have been used in bioassays. These organisms live in the sediment and, if the water is deep, will require a boat and a benthic sampling device for collection. However, it is possible in many instances to collect oligochaetes from shallow streams or lakes and polychaetes from the intertidal zone. It will be necessary to remove the specimens from the sediment as soon as possible. The use of sieves will eliminate much of the sediment but it also leads to fragmentation of the worms. Another technique is to place the sediment in shallow pans, cover it with water, and leave it undisturbed for a period of time. Specimens will crawl to the surface where they can be removed with a fine brush and placed in clean water. Examine each specimen under a dissecting microscope and discard damaged worms. Since many of these species are similar in appearance, it is necessary to make careful identifications. Laboratory culture techniques have been worked out for several species of polychaetes which will produce sufficient numbers of specimens for bioassays (Reish, 1976; 1980). Oligochaetes can be maintained for long periods of time and some can be cultured to a certain extent but thus far the techniques for routine culturing of aquatic oligochaetes have not been established. The following species have been utilized as bioassay organisms in the past. Some of the species listed are wide spread in their geographical distribution while others are restricted to an area; however, other species in the genus may serve as satisfactory test organisms.

Fresh water:
- Oligochaetes:
  - Brachiura sowerbyi
  - Limnodrilus hoffmeister
  - Tubifex tubifex

Marine:
- Oligochaetes:
  - Limnodriloides verrucosus
  - Monopylephorus cuticulatus
- Polychaetes
  - Capitella capitata - laboratory cultures
  - Ctenodrilus serratus - laboratory cultures
  - Neanthes arenaceodentata - laboratory culture. Also known under the names Neanthes (or Nereis) caudata and Neanthes (or Nereis) acuminata.
  - Neanthes succinea
  - Neanthes virens
  - Nereis diversicolor
  - Ophryotrocha spp. - many species have been cultured; specific identification difficult.

2.1.2.4 Crustaceans

Many different groups of crustaceans have been used in bioassay studies in the past including barnacles and decapod larvae, mysids, isopods, amphipods, shrimps, lobsters, and crabs. Adult barnacles can be collected by scraping the animals off rocks during low tide. The larvae lay within the mantle cavity of the adult as a thin sheet.

Remove the larval sheet, place in a dish containing sea water and examine under a dissecting microscope. If larval development has reached the cypris stage, they will soon become free swimming, which is the stage most useful for conducting bioassays.

Decapods carry the developing embryos on the ventral margin of their abdominal region until the larvae reach the free swimming stage. Collect ovigerous females from the field and isolate each one into an individual finger bowl or appropriate sized container with filtered sea water. Remove a few embryos from the adult pleopods, examine under the dissecting microscope, and determine the embryonic state. Embryos near the hatching stage have developed eyes and a visible beating heart. Examine the adult decapods each day for presence of hatched larvae. When hatching occurs, the larvae should be pipetted out, placed in an aerated aquarium, and fed Artemia nauplii. This technique has been successfully used for caridean and penaeid shrimp, anomuran and brachyuran crabs, and lobsters.

Fresh water amphipods can be collected from ponds or streams. Gammarus fasciatus has been successfully reared in the laboratory (Clemens, 1950). Marine isopods and amphipods can be collected from a variety of habitats especially during the warmer months of the year. Algal mats and mussel beds are convenient sources of large populations of these crustaceans. Examine the algae or mussels in the field to determine if a sufficient sized population is present. If so, transport the clumps of algae or mussels to the laboratory where the crustaceans can be more conveniently separated from the other organisms.

Adult decapods are collected by a variety of methods depending upon the species desired. Shrimps are generally collected by trawling, dip-net, or plankton net. Crabs and lobsters are collected from crab or lobster traps. Freshwater crayfish are collected from their habitat by hand or seine. It is important to segregate decapods into separate compartments as soon as possible because of their cannibalistic tendencies. Because of difficulties with the time involved in collection and the large laboratory space required, careful consideration should be made prior to the use of adult crabs, lobsters, or crayfish as bioassay organisms.

Many species of crustaceans have been utilized in the past as test organisms. Given below is a list of some of these. These species are not cosmopolitan in distribution; however, other species in the same genus or family may be present and could serve as suitable test organisms.

Fresh water:
- Mysidacea
  - Mysis relicta
- Amphipoda:
  - Gammarus fasciatus
  - Gammarus lacustris
  - Hyalella azteca
- Decapoda:
  - Cambarus spp.

Marine
- Cirripedia:
  - Balanus spp.
- Isopoda:
  - Idotea baltica basteri
- Amphipoda:
  - Jassa falcata
- Decapoda:
  - Callinectes sapidus
  - Cancer magister
  - Crangon spp.
  - Homarus americanus
  - Palaemonetes spp.
  - Palaemon elegans
  - Rhithropanopeus harrisii

2.1.2.5 Aquatic insects

Fresh water insects are important animals in the food chain since they provide the principle diet for many species of fish. They are abundant in streams, ponds, and lakes. Many species can be collected by disturbing the bottom by moving rocks and gravel which causes the insects to be dislodged and carried downstream by the current. Collect these animals in a net and transfer them into a container for transport to the laboratory. It will be necessary to use a bottom sampling device, such as an Ekman grab, to collect benthic insects such as chironomids, dipterans, and mayflies. Empty the sediment into a pail of water, swirl the material by hand, and decant the supernatant fluid onto a fine mesh sieve (1.0 mm opening). Repeat this process several times. Transfer the insects from the sieve into an appropriate sized bucket containing water for transporting them to the laboratory. It may be necessary to provide aeration during transportation if the distance to the laboratory is far. Given below is a list, by genus, of the species of aquatic insects which have been used in bioassays. Many of these genera are world-wide in distribution and should provide a broad basis for selection of test organisms from this group. All are fresh water species.

Diptera (two-winged flies):
- Chironomus
- Tanypus
- Tanytarus

Ephemeroptera (mayflies):
- Ephemerella
- Hexagenia
- Stenonema

Plecoptera (stoneflies):
- Acroneuria
- Isogenus
- Paragnetina
- Phasganophora
- Pteronarcys

Trichoptera (caddisflies):
- Brachycentrus
- Hydropsyche
- Macronemum

2.1.2.6 Molluscs

Many species of molluscs are utilized for human food, especially oysters, clams, mussels, and scallops. These are also the species which have been widely used as bioassay organisms. Fresh water clams and hydrobiid snails have been used in a limited number of experiments. Oysters and mussels are easily collected from estuarine waters with a minimum of effort and time. They can be placed in buckets and transported to the laboratory without any special procedures. The specimens must be cleaned of all fouling organisms before placing them in holding tanks. The byssal threads of mussels should not be pulled; however, they can be cut without causing any injury. Clams are generally dug from the sediment. Discard any specimen with broken shells. Scallops are collected by trawling. The embryos of oysters and mussels can be used in bioassays in much the same way as sea urchin larvae. The species of molluscs listed are primarily from the Northern Hemisphere; however, some of these genera and families occur in the Southern Hemisphere as well.

Oysters:
- Crassostrea gigas
- Crassostrea virginica
- Ostrea edulis
- Ostrea lurida

Mussels:
- Mytilus edulis
- Mytilus galloprovincialis

Scallops:
- Argopecten irradians

United Kingdom:
Marine Biological Association
The Hoe
Plymouth, England

Culture Centre of Algae and Protozoa
36 Storey's Way
Cambridge CB3 ODT
England

United States of America:
Algae Culture Collection
Dept. of Botany
University of Texas
Austin, Texas 78712

Scripps Institute of Oceanography
University of California
San Diego, California 92037

Woods Hole Oceanographic Institute
Woods Hole, Mass. 02543

Contact the supplier prior to the request for living material for supplies and equipment necessary to culture the species desired. The following are some of the more commonly used algae in bioassays.

Fresh water:
Chlorophyta (green algae)
Selenastrum capricornutum
Cyanophyta(blue-green algae)
Anabaena flos-aquae
Microcystis aeruginosa
Chrysophyta (diatoms)
Cyclotella sp.
Nitzschia sp.
Synedra sp.
Marine:
Chlorophyta (green algae)
Dunaliella tertiolecta
Chrysophyta (diatoms)
Skeletonema costatum
Thalassiosira pseudonana

2.1.2.2 Zooplankton

Fresh water and marine crustaceans can be collected with a fine mesh plankton net; however, because this method of collection concentrates the organisms, it will be necessary to provide aeration during transportation to the laboratory if the distance is greater than a few kilometers. Often, one species of crustacean will dominate the population of plankton. Care must be exercised to ensure that all specimens belong to the same species. Remove healthy appearing specimens with a pipette and place in a separate container for the holding period. The fresh water cladoceran *Daphnia* can be purchased from many biological supply companies. Members of the harpacticoid copepod genera *Tisbe* and *Tigriopus*, while not necessarily always inhabitants of the pelagic environment, have been used as test organisms. *Tisbe* can be collected from the surface of algae and *Tigriopus* from tide pools. Different species of the crustacean group Mysidacea have been used in bioassays. They are collected with either plankton nets or dip nets from the water surrounding offshore kelp beds. The culture methods for one species, *Mysidopsis bahia*, have been worked out. This has resulted in its use in many bioassays. The following are some of the more commonly used zooplankton in bioassays:

Fresh water:
- Cladocera
  - *Daphnia magna*

Marine:
- Copepoda
  - *Acartia tonsa*
  - *Tisbe* sp.
  - *Tigriopus* sp.
- Mysidacea
  - *Acanthomysis* sp.
  - *Metamysidopsis* sp.
  - *Mysidopsis bahia*

2.1.2.3 Annelids

Both oligochaetes, which are largely fresh water inhabitants, and polychaetes, which inhabit marine waters, have been used in bioassays. These organisms live in the sediment and, if the water is deep, will require a boat and a benthic sampling device for collection. However, it is possible in many instances to collect oligochaetes from shallow streams or lakes and polychaetes from the intertidal zone. It will be necessary to remove the specimens from the sediment as soon as possible. The use of sieves will eliminate much of the sediment but it also leads to fragmentation of the worms. Another technique is to place the sediment in shallow pans, cover it with water, and leave it undisturbed for a period of time. Specimens will crawl to the surface where they can be removed with a fine brush and placed in clean water. Examine each specimen under a dissecting microscope and discard damaged worms. Since many of these species are similar in appearance, it is necessary to make careful identifications. Laboratory culture techniques have been worked out for several species of polychaetes which will produce sufficient numbers of specimens for bioassays (Reish, 1976; 1980). Oligochaetes can be maintained for long periods of time and some can be cultured to a certain extent but thus far the techniques for routine culturing of aquatic oligochaetes have not been established. The following species have been utilized as bioassay organisms in the past. Some of the species listed are wide spread in their geographical distribution while others are restricted to an area; however, other species in the genus may serve as satisfactory test organisms.

Fresh water:
- Oligochaetes:
  - *Brachiura sowerbyi*
  - *Limnodrilus hoffmeister*
  - *Tubifex tubifex*

Marine:
- Oligochaetes:
  - *Limnodriloides verrucosus*
  - *Monopylephorus cuticulatus*
- Polychaetes
  - *Capitella capitata* - laboratory cultures
  - *Ctenodrilus serratus* - laboratory cultures
  - *Neanthes arenaceodentata* - laboratory culture. Also known under the names *Neanthes* (or *Nereis*) *caudata* and *Neanthes* (or *Nereis*) *acuminata*.
  - *Neanthes succinea*
  - *Neanthes virens*
  - *Nereis diversicolor*
  - *Ophryotrocha* spp. - many species have been cultured; specific identification difficult.

2.1.2.4 Crustaceans

Many different groups of crustaceans have been used in bioassay studies in the past including barnacles and decapod larvae, mysids, isopods, amphipods, shrimps, lobsters, and crabs. Adult barnacles can be collected by scraping the animals off rocks during low tide. The larvae lay within the mantle cavity of the adult as a thin sheet.

Remove the larval sheet, place in a dish containing sea water and examine under a dissecting microscope. If larval development has reached the cypris stage, they will soon become free swimming, which is the stage most useful for conducting bioassays.

Decapods carry the developing embryos on the ventral margin of their abdominal region until the larvae reach the free swimming stage. Collect ovigerous females from the field and isolate each one into an individual finger bowl or appropriate sized container with filtered sea water. Remove a few embryos from the adult pleopods, examine under the dissecting microscope, and determine the embryonic state. Embryos near the hatching stage have developed eyes and a visible beating heart. Examine the adult decapods each day for presence of hatched larvae. When hatching occurs, the larvae should be pipetted out, placed in an aerated aquarium, and fed Artemia nauplii. This technique has been successfully used for caridean and penaeid shrimp, anomuran and brachyuran crabs, and lobsters.

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- Phasganophora
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- Crassostrea virginica
- Ostrea edulis
- Ostrea lurida

Mussels:
- Mytilus edulis
- Mytilus galloprovincialis

Scallops:
- Argopecten irradians

Clams:

Macoma baltica
Macoma nasuta
Mercenaria mercenaria
Mulinia lateralis
Rangia cuneata

2.1.2.7 Echinoderms

Sea urchins provide the most satisfactory source of eggs and larvae among the echinoderms for bioassays. Sea urchins can be collected by hand during low tides; however, in some parts of the world it may be necessary to collect them from subtidal waters either by SCUBA or by trawling. Sea urchins are usually sensitive to both high and low temperatures so some method of temperature control is necessary. Determine the reproductive season of the more abundant species of sea urchins in the area in order to plan what times of the year such bioassays can be conducted. Undoubtedly many intertidal and shallow water species of sea urchins would provide an adequate supply of gametes; the species listed have been extensively studied.

Arbacia punctulata
Echinus esculentus
Lytechinus pictus
Strongylocentrotus franciscanus
Strongylocentrotus purpuratus

2.1.2.8 Fish

Because of their economic importance, at least one species of fish should be used as a test organism whenever a battery of bioassays are conducted. In addition, many species of fish are sensitive to pollutants and are good candidates for bioassays. Since many fresh water species are reared in governmental or private hatcheries, obtain specimens from them rather than collect them from natural waters. However, in some cases it may be necessary to collect fish from the natural environment. Fish can be collected with the use of dip-nets or seines. The fish must be transferred to a container of water as soon as possible for transport to the laboratory. Depending upon the climatic conditions, it may be necessary to provide temperature control and aeration during transportation. At the laboratory, transfer the specimens into appropriately sized aquaria as soon as possible for the holding period.

Since very few marine fish are cultured, it will be necessary to collect these fish from the natural environment. Some smaller species can be collected with dip-nets. In many instances it may be necessary to trawl for fish in offshore waters. Each trawl should be of short duration so as to minimize the amount of damage to the fish. Transport and handle marine fish as outlined for fresh water fish.

Given below are names of fish listed by habitat and family which have been utilized as test organisms in the past. While none of these are cosmopolitan in distribution, the list serves as a guide for selection of appropriate species.

Fresh water:

Clupeidae
Alosa pseudoharengus

Salmonidae
Coregonus spp.
Oncorhynchus spp.
Salmo spp.
Salvelinus fontinalis

Ictaluridae
Ictalurus spp.

Cyprinidae
Carassius auratus
Cyprinus carpio
Pimephales promelas

Gasterosteidae
Gasterosteus aculeatus

Percichthyidae
Morone chrysops

Poeciliidae
Gambusia affinis
Poecilia reticulata

Percidae
Perca spp.

Centrarchidae
Lepomis macrochirus
Micropterus spp.
Pomoxis spp.

Marine Water:

Clupeidae
Brevoortia sp.
Clupea harengus

Cyprinodontidae
Cyprinodon variegatus
Fundulus spp.

Atherinidae
Atherinops affinis
Menidia sp.

Percichthyidae
Morone saxatilis

Mugilidae
Mugil spp.

Pleuronectidae
Pseudopleuronectes americanus

Engraulidae
Anchoa spp.

2.1.3 Laboratory maintenance of test organisms

Transportation of field collected animals to the laboratory is a crucial phase of bioassays. Organisms may have been injured during collection, they may have parasites, and they are generally crowded in the containers during transit. At the laboratory, transfer healthy animals into aquaria containing clean water at a similar temperature. Discard injured or parasitized specimens. If 10-20% of the field collected animals die within a day or two of collection, do not use the remaining ones in final bioassays. They can, however, be used in preliminary, range-finding type of test. Keep field collected animals under daily observation for a minimum of seven days before use in bioassays.

2.1.3.1 Environmental conditions

In general, maintain the animals in similar environmental conditions as present in the field. Water temperature changes should be minimal and not greater than 1-3°C within a 24-hour period. Dissolved oxygen content of the water should be at least 5.0 mg/l. Water quality should be similar to natural waters in so far as possible.

2.1.3.2 Water supply

Fresh water will vary from one locality to the next and from one municipal water supply to the next. It is necessary to have a constant, dependable, good quality water supply for use in fresh water bioassays. Fresh water will vary according to the contained salts (hardness), pH, chlorine, dissolved inorganic and organic compounds, as well as potential pollutants such as pesticides. A water analysis should be made to determine whether or not there is any contamination. If municipal water is used, it should be aerated for at least 24 hours before use to eliminate possible chlorine residues. If a supply of good quality fresh water is not available, it may become necessary to transport it from where the test animals were collected. It will be necessary to filter the water soon after collection to eliminate microscopic organisms and suspended sediment. Collect a quantity of water at one time sufficient to complete the bioassay.

If the bioassays are conducted at a marine station, there is generally an adequate supply of seawater to maintain the test organisms. Since the degree of filtration at marine stations varies considerably, it may be necessary to re-filter the water to remove microorganisms and suspended sediment. If the source of the sea water is from subtidal open oceanic waters, the salinity should remain relatively constant. If, on the other hand, the source of the sea water is from an estuary, the salinity may show considerable variation especially during the rainy season. The salinity of the water should be analyzed as a routine laboratory practice. Depending upon the species of test organism used, the salinity should not vary more than 1.0 °/oo S during the holding period. If a good supply of sea water is not available, it may be necessary to either

purchase sea salts or prepare artificial sea water (Table I). In either case, it will be necessary to filter the artifical sea water to remove undissolved chemicals prior to use.

Table I

Chemical composition of artificial sea water (35.00 °/oo S) (from Kester et al., 1967. Reproduced with kind permission of American Society of Limnology and Oceanography. © 1967).

Chemical	Molecular Weight	Amount
NaCl	58.44	23.926 g/kg of solution
Na_2SO_4	142.04	4.008 "
KCl	74.56	0.677 "
$NaHCO_3$	84.00	0.196 "
KBr	119.01	0.098 "
H_3BO_3	61.83	0.026 "
NaF	41.99	0.003 "
$MgCl_2 \cdot 6H_2O$	203.33	10.83 "
$CaCl_2 \cdot 2H_2O$	147.03	1.52 "
$SrCl_2 \cdot 6H_2O$	266.64	0.02 "
H_2O (Distilled)	16.0	to 1,000 g

2.1.3.3 Phytoplankton

Since laboratory cultured species of phytoplankton are often used as test organisms, a brief description of the culture procedures is included herein. The reader, however, is directed to "Standard Methods" (APHA/AWWA/WPCF, 1985) for a more complete account. The technique for culturing the marine species Dunaliella tertiolecta is outlined below as an example for growing a phytoplanktonic species. The techniques for other species are basically similar to those described for Dunaliella.

Preparation of culture medium

Seawater medium (A)

1. Use 30-32°/oo S sea water; dilute with double distilled water if necessary to lower salinity or add sea salts to increase the salinity.

2. Filter through a 45 µ microporous filter system.

Nitrate solution (B)

1. Nitrate. Add 5 g KNO_3 to distilled water to make a 1000 ml solution. Store in refrigerator for maximum of four months before discarding. Take 1.0 ml of stock nitrate solution and add to 999 ml distilled water to make final solution.

Phosphate solution (C)

Add 0.68 g KH_2PO_4 to distilled water to make a 1000 ml solution. Store in

refrigerator for maximum of four months before discarding. Take 1.0 ml of stock phosphate solution and add to 999 ml distilled water to make final solution.

Trace metal mixture (D)

1. Add 30 mg $ZnSO_4 \cdot 7H_2O$; 25 mg $CuSO_4 \cdot 5H_2O$; 20 mg $CoSO_4 \cdot 7H_2O$ to distilled water to make a 1000 ml solution.

2. Dissolve 5.0 g $FeCl_3 \cdot 6H_2O$ and 2.0 g $MnSO_4$ in 1000 ml distilled water; ignore slight precipitate.

3. Dissolve 25 mg $Na_2MoO_4 \cdot 2H_2O$ in 1000 ml of distilled water.

4. Dissolve 50 g of sodium ethylene diamine tetra acetate (EDTA) in 1000 ml distilled water.

5. Add 100 ml of EDTA solution and 10 ml of each of the three trace metal solutions listed above to 800 ml of distilled water.

6. Adjust to pH 7.5 with dilute NaOH and add distilled water to 1000 ml.

Vitamin B_{12} (E)

1. Dissolve 10 mg crystalline vitamin B_{12} in 100 ml distilled water.

2. Store in freezer until needed.

3. Immediately prior to use, thaw the solution, remove 1.0 ml, and refreeze the stock solution.

4. Dilute the 1.0 ml of vitamin B_{12} solution with 99 ml of distilled water.

Biotin (F)

1. Dissolve 10 mg biotin in 100 ml distilled water.

2. Store in freezer until needed.

3. Immediately prior to use, thaw the solution, remove 1.0 ml and refreeze the stock solution.

4. Dilute the 1.0 ml of biotin solution with 99 ml of distilled water.

Thiamine hydrochloride (G)

1. Dissolve 100 mg of thiamine hydrochloride in 100 ml of distilled water.

2. Store in freezer until needed.

3. Immediately prior to use, thaw the solution, remove 1.0 ml and refreeze the stock solution.

4. Dilute the 1.0 ml of thiamine hydrochloride solution with 99 ml of distilled water.

Mixing of culture medium

1. Add to 1 litre of seawater medium (A)

   10 ml nitrate solution (B)
   1 ml trace metal mixture solution (D)
   1 ml vitamin B_{12} solution (E)
   1 ml biotin solution (F)

1 ml thiamine hydrochloride solution (G)

Autoclave this mixture (H)

2. Autoclave phosphate solution (C)

3. Add 10 ml of autoclaved phosphate solution to autoclaved mixture (H)

4. If precipitate occurs, it may be necessary to filter prior to use.

Note: Remainders of solutions (B,C,E,F,G) must be discarded. Only the trace metal mixture (D) can be stored for up to 4 months in a refrigerator.

Culture

Mix complete medium and autoclave this and the phosphate solution separately. Mix together; if precipitate occurs it may be necessary to filter prior to use.

Place 5-15 ml of Dunaliella from a previous culture in a 500 ml Erlenmeyer flask containing 500 ml of autoclaved medium. The amount of Dunaliella depends upon its concentration in the previous culture. Place stoppered Erlenmeyer flasks under grow-lux fluorescent lights. Depending upon the amount of Dunaliella required, the algae can be exposed to continuous light or to a light-dark cycle, which is maintained with an electrical timer. The growth rate can be slowed, if desired, by placing the cultures in a cold bath (17°C) with the lights placed overhead.

2.1.3.4 Zooplankton

While the actual holding and maintenance procedures will vary from species to species, there are certain techniques which will apply to most species of pelagic crustaceans. In view of the large number of marine crustaceans, which are listed under section 2.1.2.4, it would be well to concentrate on one to three species which are convenient to collect and easy to maintain in the laboratory. Many smaller glass aquaria of 1-4 litre capacity are preferred to one larger aquarium. Aeration is generally provided, but in some cases sufficient dissolved oxygen is given off by such food as the green alga Ulva sp. when smaller containers are used.

Daphnia magna - Place 100 ml of either natural water or dechlorinated tap water in a 150 ml flask. Add one female Daphnia to the container together with a small quantity of food. Daphnia can be fed a manure-soil medium, phytoplankton such as Dunaliella, or a mixture of powdered grass and trout fry granules (Buikema et al., 1980; APHA/AWWA/WPCF, 1985). Manure-soil food is prepared by mixing 5 g of sheep manure and 25 g of garden soil in 1 litre of water. After two days filter through a fine sieve and discard the material retained on the sieve. The filtrate can be kept indefinitely. Mix 88 ml water to 11 ml filtrate for use as a final medium. Add 1.0 ml of a yeast suspension to the culture every other day. A parent population will produce 300 young Daphnia per day. When phytoplankton is used as a food source, add 1.0 ml as needed. Powdered grass (0.1 g) and trout fry granules (2.0 g)are mixed at a 1:20 ratio in a blender containing 50 ml of water. It is stored in a refrigerator until needed. Cultures are fed at the rate of 1.0 ml per litre each week.

The marine copepods Acartia tonsa, Tisbe sp., and Tigriopus sp. can be cultured in 20 l to 50 litre aquaria. Aeration must be provided. Acartia tonsa is fed a mixture of four species of phytoplankton: Skeletonema costatum, Thalassiosira pseudonana, Isochrysis galbana, and Rhodomonas baltica. Tigriopus is fed 10 ml of a powdered TetraMin suspension per week. Grind up approximately 0.01 g of TetraMin into a fine powder and place in a 500 ml Erlenmeyer flask together with 500 ml of sea water. Shake the flask and allow 3-5 minutes for the suspension to settle. Use the supernatant suspension for feeding Tigriopus. Tisbe sp. is fed Dunaliella, or small pieces of Ulva sp. together with boiled wheat. A continuous supply of copepods will be provided if the cultures are carefully monitored on a more-or-less daily basis.

The various species of marine mysids can be cultured for several generations in 25-50 litre aquaria provided with aeration. Newly hatched Artemia nauplii larvae provide

satisfactory food for mysids.

Diseases. The most common problem with maintaining or culturing these planktonic crustaceans is the lack of regular, systematic care. While parasites undoubtably occur in copepods, they have not received attention in laboratory populations. Many different parasites, especially protozoans, copepods, and isopods, have been reported from mysids (Mauchline, 1980), but it is not known if they cause any problems in laboratory colonies. Any specimen observed with a parasite should be discarded. Crustacean cultures often lose vitality after a few generations so that it will be necesary to establish new cultures periodically.

2.1.3.5 Annelids

The procedures for maintaining and culturing oligochaetes and polychaetes have been well established for a number of species. A large number of species can be obtained on a regular basis with a minimum of time and space (Reish, 1980; Chapman, et al., 1982). Some species of polychaetes can be cultured in Petri dishes while others may require 4 to 50 litre aquaria which require aeration. Oligochaetes must be either kept in the dark or under natural light/dark conditions. Polychaetes do not require any special lighting regime. While most annelids are grown as pure cultures, some oligochaetes do better in mixed cultures, such as species in the genera Limnodrilus, Tubifex, Brachiura, and Stylodrilus.

Food. Many different types of food have been used in culturing annelids but the tendency has been towards utilization of prepared foods or dried algae. Many species of oligochaetes can be maintained and cultured in natural sediments without adding additional food. Sediments can be sterilized in an autoclave to which an inoculum of decayed lettuce, for fresh water species, or Enteromorpha for marine species, is added for food. Since oligochaetes lack a larval stage, no special handling of juveniles is required.

Many species of nereid polychaetes can be fed a variety of dried plants as food, such as Enteromorpha or commercially prepared alfalfa flour. Enteromorpha is collected from estuarine mud flats, washed, and dried for storage. Prior to feeding, the alga is resoaked and kneaded in sea water which separates the individual filaments and prevents fungal growth. Fish flakes, such as TetraMin, are placed in a blender with sea water and blended. The mixture is allowed to stand 1-2 hours after which the supernatant is drawn off and used as food. Akesson (1970) utilizes frozen spinach as food for species of the genus Ophryotrocha which is processed in the same manner as described for TetraMin. Planktonic larval stages of polychaetes as well as filter-feeding adult polychaetes of such families as Sabellidae or Serpulidae can be fed Dunaliella tertiolecta or other species of phytoplankton.

Diseases. Difficulties with maintaining populations of polychaetes and oligochaetes generally arise from lack of routine care. Overfeeding may result in the decrease in the dissolved oxygen concentration leading to the formation of fungal growth over the aquarium bottom. Annelids generally posses gregarine protozoan parasites. In most instances it is not known whether these have harmful effects on the host; however, Akesson (1970) has noted that gregarines reduce the vitality of laboratory populations of Ophryotrocha spp. over a period of time.

2.1.3.6 Crustaceans

Fresh water and marine crustaceans are fed a variety of foods. Larval stages of barnacles and decapods are initially fed one or more species of phytoplankton, then Artemia nauplii. Adult barnacles are also fed Artemia nauplii. Fresh water amphipods are fed tree leaves which have been soaked in flowing water for several weeks and commercial fish food. Marine amphipods and isopods can be fed a variety of foods such as pieces of the dried green algae Codium, Ulva, the brown alga Sargassum, or commercial fish food. Foods for fresh water and marine decapods include cut up or macerated fish, clams, mussels, or dried fish food. A certain amount of trial and error effort will be necessary in order to determine what type(s) of food are best suited to the species being maintained. Regardless of the type of food fed to these crustaceans, it is important that they are not overfed and that the holding containers be kept clean to

prevent bacterial and fungal growth. Furthermore, since most crustaceans, except barnacles, are cannibalistic, they must be kept in separate containers or in large enough aquaria that each specimen has its own territory.

Diseases. In general, the larger the crustacean the greater the possibility of having outbreaks of diseases in the holding containers. Bacterial and fungal infections are the most commonly encountered diseases which are caused by overfeeding, low dissolved oxygen concentration in the water, or the presence of one or more dead specimens in the container. If such conditions occur, it is usually necessary to destroy all the animals and sterilize the container. None of these animals should be used as test organisms except possibly in a preliminary range-finding test. A common bacterial infection causes a shell disease that destroys the chitin of the crustacean. If bacterial and fungal growths are a repeated problem, then it may become necessary to do one of the following: (1) filter the water through a micropore filtration system, (2) use artificial sea salts, (3) use antibiotics, or (4) sterilize the water with ultraviolet radiation. Larval stages of many different types of parasitic worms utilize crustaceans in their life cycle. It is not known whether or not these have any adverse effects on the crustacean hosts. Isopods and barnacles occur on the gills of decapods which may cause a reduction in the vitality of the host. As a general rule, do not use parasitized specimens. If heavy internal parasitism is suspected, examine some of the animals internally to determine whether or not larval parasites are present. If many of the specimens are parasitized, do not use these specimens in final bioassay tests.

2.1.3.7 Aquatic Insects

Food. These animals are fed a variety of foods. Given below are those genera which were listed in section 2.1.2.5 and their laboratory diet. It will be necessary to determine foods for genera not on this list; however, the different foods listed could serve as a convenient guide for selecting food for different aquatic insects.

Diptera (chironomids). Maintain them in aquaria with a thin layer of silt on the bottom. Feed them with a mixture of phytoplankton and diatoms. Periodically feed them fish food and powdered dry grass.

Ephemeroptera and some Plecoptera (Pteronarcys). Chopped dried leaves from such trees as maple, aspen, or birch which have been soaked in running fresh water for two weeks.

Many Plecoptera and some Trichoptera - these forms require live food such as larvae of midges, blackflies, or mosquitoes. These larvae can be collected from small ponds or fish hatcheries with a fine net.

Trichoptera (Hydropsyche). They can be fed a mixture of fish pellets and chopped leaves.

Diseases. Any diseases which might occur are the result of overfeeding, poor water quality, or dirty containers. These factors can lead to microbial or fungal growths. Some aquatic insects, such as members of the genus Hydropsyche, are cannibalistic especially if they are underfed. Since they require live animal food, there is less danger of microbial growths occurring in this genus.

2.1.3.8 Molluscs

Food. It is not necessary to feed pelecypods if they are used within 7 to 10 days after collection. It is important, however, to examine the holding tanks daily for deaths and spawning. Most larvae of pelecypods will not require feeding since they contain a sufficient supply of stored yolk to sustain them for more than the 96 hour test period.

Diseases. Poor water quality in the holding aquarium leads to microbial growth. This is generally the result of the death of attached organisms on the shells, the accumulation of fecal material, overcrowding, or decomposition of released gametes. The aquarium must be kept clean and, if possible, provided with running water. Cyclopoid copepods are frequently present on the gills or in the mantle cavity. These organisms

are not known to cause any hardship to the pelecypod. Other cyclopoid copepods, such as Mytilicola intestinalis, occur in the digestive tract of some pelecypods, such as oysters, and can cause damage to the cellular lining of the gut (Cheng, 1967). Examine the stomach or intestine if the presence of this parasite is suspected or if the animals were collected from a region where it is known to occur. Discard all specimens if the incidence of infection is greater than 10%.

2.1.3.9 Echinoderms

Since sea urchins are the primary source of gametes used in embryo bioassays, only those techniques used in maintaining these animals will be discussed. If species in other classes of echinoderms are utilized as a source of gametes, it may be necessary to first determine how to maintain them under laboratory conditions prior to experimentation.

Food. Adult sea urchins can be fed a variety of seaweeds such as the brown algae Macrocystis, Egregia, Laminaria, Fucus, or the green alga Ulva. The algae used as food should be in good condition and free of attached organisms. Adult Lytechinus requires about 25 cm^2 of algae per individual and feeding (Hinegardner and Tuzzi, 1981). Leafy vegetables such as lettuce have also been used for feeding adult sea urchins. Sea urchin embryos do not require feeding until they reach the pluteus larval stage. Since the pre-pluteus larvae are the common stage used in bioassays, feeding is unnecessary. See Hinegardner and Tuzzi (1981) for details on feeding and maintaining pluteus larvae.

Diseases. Healthy adult sea urchins can be maintained in the laboratory for long periods of time provided they have sufficient food, aquaria are kept clean and free of debris, water is filtered and aerated, and some method of temperature control is employed. No specific diseases are known, but rapidly growing microbial growths may occur as the result of inadequate care or the presence of dead sea urchins. This will result in a weakening or death of all specimens.

2.1.3.10 Fish

Many books have been written on the care and feeding of fish. For fresh water fish consult such references as Hunn et al. (1968), National Academy of Sciences (1973), and Hokanson et al. (1973). If freshwater fish are obtained from hatcheries, follow the same maintenance procedures in the laboratory as used at the hatchery as much as possible. References by May (1970), Houde and Palko (1970), and Boyd and Simmons (1974) are useful for maintaining marine species.

Food. Fresh water fish can generally be maintained on dried commercial fish food; however, an occasional feeding with living food such as zooplankton or sludge worms (Tubifex) may increase their vitality. Marine fish, such as Cyprinodon variegatus, can be fed either fresh or frozen brine shrimp. Other species, such as Menidia, can be fed chopped mussels or fish.

Diseases. Fish are very susceptible to various diseases and parasites. Each fish brought into the laboratory should be examined carefully for injuries, diseases, and external parasites; any such fish should be removed and discarded. Fish should be observed daily during the acclimation period and any dead, injured, or sick fish should be removed. Fish should not be crowded, overfed, and the aquaria kept clean in order to minimize the appearance of diseases. The treatment of diseased fish is further advanced than for the other marine animals used in bioassays. Many reviews on this subject have been written, some of these are: Snieszko (1970), van Duijn (1973), Hoffman and Meyer (1974), and Sindermann (1970).

2.2 Laboratory

The general laboratory requirements were described under section 2.1.1.1. An air-conditioned laboratory measuring approximately 100 m^2, equipped with tables, shelves, water supply, etc., should provide adequate space in which to maintain test organisms and conduct bioassays.

2.2.1 Temperature conditions

In most instances the laboratory temperature will be higher than the environment from which the animals were collected; this necessitates some method of temperature control. Constant temperature conditions can be maintained with air conditioning, construction of a small cold room, or by refrigerated aquaria or cold baths. An air conditioned laboratory is the most desirable since this will afford the greatest amount of space; however, such a facility will be difficult to maintain if the temperature requirements for the test organisms is less than 15°C. Temperature should not fluctuate more than 1-2°C during a test period.

2.2.2. Lighting

The lighting requirements will vary according to the needs of the organisms. Phytoplankton, of course, will require lights in order to carry on photosynthesis as indicated in section 2.1.3.3. Some animals, such as many crustaceans or fish, require a more-or-less definite light-dark regime. Others, such as polychaetes, do not have any special light requirements. Most light regimes are scheduled according to the natural photoperiods. If the laboratory has windows it may not be necessary to provide artificial lighting for the animals. If, however, the laboratory lacks windows, then cool fluorescent lighting should be provided. The cycle should be controlled by an electric timer set to the natural day light cycle for that locality unless the species has different light requirements.

2.2.3 Aeration

It is necessary to supply air to the animals during the acclimation period and to some during the bioassay. A single large compressed air pump is more satisfactory than many small aquarium pumps. The compressed air can be distributed through a system of PVC pipes fitted with individually controlled valves. These valves, in turn, are fitted with plastic tubing which channels the air to large aquaria or to additional valves and smaller sized aquaria. An oil trap filtration system may be necessary if oil droplets appear in the plastic tubing.

2.2.4 Equipment and supplies

Given below is a list of equipment and supplies needed to carry out 96 hour bioassays with a variety of test organisms. It is not necessary to have some items of equipment listed so long as they are easily available in another laboratory. If a particular supply is unavailable, it is possible, in some instances, to improvise and use another item in its place.

1. Analytical balance
2. Autoclave
3. Atomic absorption spectrophotometer
4. Gas chromatograph
5. Dissecting microscope
6. Compound microscope
7. pH meter
8. Fluorometer to measure chlorophyll *a*
9. Haemocytometer
10. Ocular micrometer
11. Glass or plastic Petri dishes
12. 125 and 250 ml Erlenmeyer flasks
13. Centrifuge and tubes
14. Filter paper, including membrane filters (0.45 µ pore size)
15. Pipettes, various sizes
16. Rotary shaker
17. Particle counter, such as a Coulter counter
18. Compressed air system equipped with plastic tubing, air stones, and glass tubing
19. Aquaria ranging in size from 4-100 litres or larger (holding ponds) for fish.

2.2.5 Procedures for cleaning glassware

Some toxicants will adhere to the surface of used test containers and glassware and, if the container (either glass or plastic) is used again, there is a possibility of contamination. Therefore, it is extremely important that all used test containers and glassware be cleaned according to the procedures given below. In some instances, it may be more convenient or economical (considering labor costs) to discard the containers.

1. Scrub with a detergent
2. Rinse five times with tap pwater
3. Rinse with 10% HCl or HNO_3. If metals were used, soak glassware in an acid bath for at least 24 h.
4. Rinse five times with tap water
5. Rinse five times with either distilled or deionized water.
6. Dry in a dust free atmosphere
7. If available, wash pipettes in an automatic pipette washer.

2.3 Test Solution

Prepare test solutions immediately prior to initiation of the experiment. Make certain that all chemicals are in solution, or, if this is not possible, have them evenly dispersed throughout the solution. If a precipitate forms, either discard the solution or filter the solution to remove the precipitate. However, if filtration is used, the concentration of the solute must be verified by analytical methods (i.e. atomic absorption spectrophotometry or gas chromatography). Do not use a solvent to dissolve the chemical unless it is absolutely essential, and, if one is used, do not add more than 0.5 mg/l. Whenever a solvent is used, conduct a second control series with the solvent alone at the highest concentration employed.

If bioassays are conducted with effluents, do not alter by aeration, filtration, or addition of chemicals. Disperse undissolved material with a stirring device just prior to withdrawing subsamples. The chemical composition of effluents need to be analyzed for toxicant concentrations.

2.3.1 Selection of test concentrations

If the toxicant is available in solid form, such as a metallic salt, the concentrations of test solutions should be selected from a logarithmic scale (Table II). Each number within a column represents a concentration and each of these numbers bisects two concentrations in the immediate left hand column. The test concentrations can be selected from Table II based on the range-finding bioassays. The test concentrations may be widely separated, such as 1.0, 3.2, 10.0, 32, etc., or close together such as 1.0, 1.35, 1.8, 2.4, 3.2, 4.2, 5.6, 7.5, 10.0, etc. depending on the toxicity of the compound. If, on the other hand, the toxicant being tested is an effluent, then dilutions can be made using the concentrations given in Table II as, for example, 100, 75, 56, 42, 32, 24, 18, 13.5, and 1%. Each number within these examples is evenly spaced apart on a logarithmic scale.

2.3.2 Preparation of test solutions

If the toxicant being tested is in solid form, such as a metallic salt, a stock solution should be made. This solution should be approximately two orders of magnitude greater than the highest test solution (i.e. if the highest test concentration is 10 mg/l, then the stock solution should be 1000 mg/l). This minimizes errors due to weighing and making dilutions. However, modifications may be required because of the low solubility of some compounds. The metallic salt should be mixed in double distilled water and thoroughly stirred. Consult the procedures outlined in section 5.1.3 for serial dilutions from stock solutions of metallic salts and section 5.5 for preparation of dilutions from liquid effluents.

2.3.3 Analysis of solutions

Because of potential errors in weighing materials and mixing solutions, it is important for accuracy to verify the concentration of the toxicant in the stock solution of test concentrations by analytical methods. Concentrations of heavy metals are measured by atomic absorption spectrophotometry, and gas chromatogaphy is used for analyzing petroleum and pesticide solutions. Consult FAO/SIDA (1983) for procedures in analyzing with these instruments. If the actual and calculated concentration of the toxicant differ, then the concentration for the stock solution must be adjusted to the measured value.

Since contaminated sediments and effluents are collected from the field, the nature and concentration of the potential contaminants is unknown. It is essential that a chemical analysis be made of the sediments and effluents. Unless the specific contaminant is known, it will probably be necessary to use both atomic absorption spectrophotometry and gas chromatography to measure the concentration of toxicants in the sediments and effluents.

Table II

Guide to the selection of test concentration based on a logarithmic scale

(From APHA/AWWA/WPCF, 1976. Reproduced with kind permission of the American Public Health Association, © 1976)

Test Concentrations in mg/l, mg/g, or percent				
Column 1	Column 2	Column 3	Column 4	Column 5
10.0				
				8.7
			7.5	
				6.5
		5.6		
				4.9
			4.2	
				3.7
	3.2			
				2.8
			2.4	
				2.1
		1.8		
				1.55
			1.35	
				1.15
1.0				

2.4 The Experiment

2.4.1 Examination of test organisms

All potential test organisms should be examined for condition. If many specimens are dying at the end of the holding period, either discard all specimens and collect a new population or keep the surviving ones for an additional holding period of one week. If specimens are still dying at the end of the second holding period, discard all specimens and collect a new population. If the animal is small, such as a polychaete or an amphipod, examine each specimen under a dissecting microscope for possible injury, or presence of developing ova (polychaetes) or carrying embryos (amphipods); do not use if

so in either of these cases. If the animal is large, such as a clam or fish, make certain that they are in a healthy condition. Healthy, undisturbed clams usually have their shells ajar and are pumping water through their bodies. Healthy fish will be swimming in a normal manner. Transfer the healthy organisms into appropriate sized containers as indicated for each type of organism in Section 4.

2.4.2 Replication

Use a minimum of 10 organisms per concentration for larger species and 20 for smaller species. It is advantageous to have three replicates per concentration so the 95% confidence limits can be determined for each replicate series and the calculated LC50's can be statistically compared to determine whether or not there are significance differences between replicate series (see Section 3 for data analysis).

2.4.3 Random placement of test organisms

Place healthy organisms in appropriate sized containers lacking toxicant. The organisms should be randomly selected for a particular concentration series. To accomplish this, number each container consecutively. Selection of test organisms for a particular concentration and replicate can be made by following a random numbers table (Zar, 1974) or by drawing the numbers from a hat. In either case, the first one chosen could be in the control series - replicate 1, organism 1; the second, control series - replicate 2, organism 1; the third, control series - replicate 3, organism 1; the fourth, lowest concentration series - replicate 1, organism 1, etc.

2.4.4 Observations

Examine each specimen for death and record the observations daily for the 96 hour test period. Death is the measure generally used in 96 hour tests. Death may be difficult to determine in some of the species. Lack of movement is the most frequently used criterion for death. Lack of opercular movement in fish is an indication of pending death in fish. Experience in determining death will be gained during the holding, pre-test period as well as the test period itself. Record indications of stress or abnormal behavior in the test organisms; they are generally forerunners of death and will provide additional data from the experiment.

2.4.5 Control

The control series is an essential part of any experiment. There must be a control series with the same number of organisms and replicates as in each experimental series. If a chemical solvent is used to get a toxicant into solution, then a second control series containing the solvent is also required. Repeat the experiment if more than 10% of the specimens in the control die.

2.4.6 Conclusion of bioassay

Record the number of living and dead organisms in each of the test series and replicates. The 96 hour mortality data will be utilized in calculating the 96 hour LC50 for each replicate series. The test solutions should be disposed of in a safe manner. While it is advantageous to use the test container only once, this is not usually economically feasible. Test containers must be scrubbed and washed thoroughly with a chemical cleaner to remove all traces of the toxicant. See Section 2.2.5 for methods of cleaning test containers contaminated with toxicants.

3. DATA ANALYSIS

Many different types of data analysis are available to assist in the interpretation of the results. Some of these statistical treatments are easily performed without a hand calculator. Others require additional time which can be greatly assisted by using a hand calculator or computer. However, all examples given below can be performed without a computer.

3.1 Data Analysis for 96 Hour LC 50

The results of a hypothetical bioassay with three replicates are presented in Table III. All statistical calculations made in the various analyses subsequently described are based on the data given in this table.

3.1.1 Arithmetic graphic method

Graph paper is used in this type of analysis (Fig. 1) with per cent survival plotted on the ordinate against the concentration on the abscissa. Each data point (Table III) is plotted and connected to form a graph. A horizontal line is drawn from the 50 percent survival point to intersect the plot. A verticle line from the intersection point is then dropped to the abscissa. This intersection point on the abscissa corresponds to the 96 hour LC50 (Fig. 1). A graph for each of the three replicates is presented in Figure 1. The 96 hour LC50s for this example were 2.8, 2.6, and 2.7 mg/l for replicates 1, 2, and 3, respectively. These 96 hour LC50 results may be presented separately or as an average of the three, which in this example would be 2.7 mg/l.

Table III

The effect of a toxicant on an animal
(20 animals/concentration)

Concentration (mg/l)	Number of Survivors at 96 hours		
	Replicate 1	Replicate 2	Replicate 3
Control	20	20	20
1.0	16	19	20
1.8	13	12	12
3.2	9	8	9
5.6	7	7	6
10.0	2	1	0

3.1.2 Logarithmic method

This technique of LC50 calculation is similar to the arithmetic method except that semi-log paper is used for graphing the results (Fig. 2). The concentration is plotted logarithmically along the ordinate axis. This method of calculation is preferred because the concentrations used are based on a logarithmic scale. The 96 hour LC50s using the logarithmic method were 2.8, 2.4 and 2.6 mg/l for replicates 1, 2, and 3, respectively (Fig. 2) for an average value of 2.6.

3.1.3 Probit method

This method of calculation of the LC50 minimizes the extremes in the results and maximizes the middle percentage survivals. Using the data given in Table III, the percent mortality for each replicate is calculated and given in Table IV. The percent mortality versus concentration are plotted on probit graph paper for the three replicates in Figure 3. Note that probit graphs neither have a zero nor 100 point. Therefore, the data for all three controls, 1.0, and 10.0 mg/l in replicate 3 do not appear on the graph. A straight line is drawn through the remaining data points, especially those in the 40-60% mortality range.

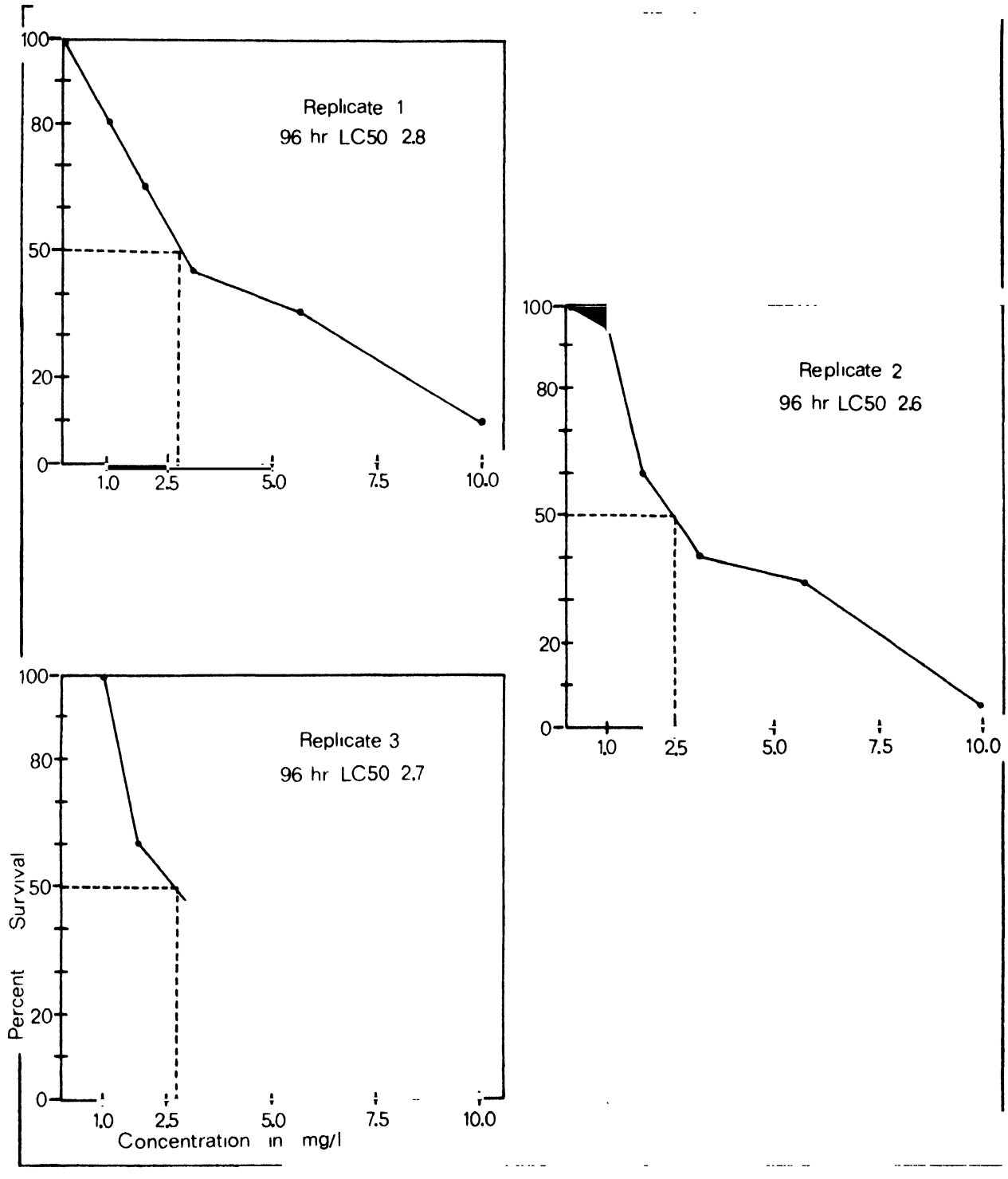

Figure 1. Calculations of the 96 hour LC50 by the arithmetic graphic method for 3 replicates (Table III).

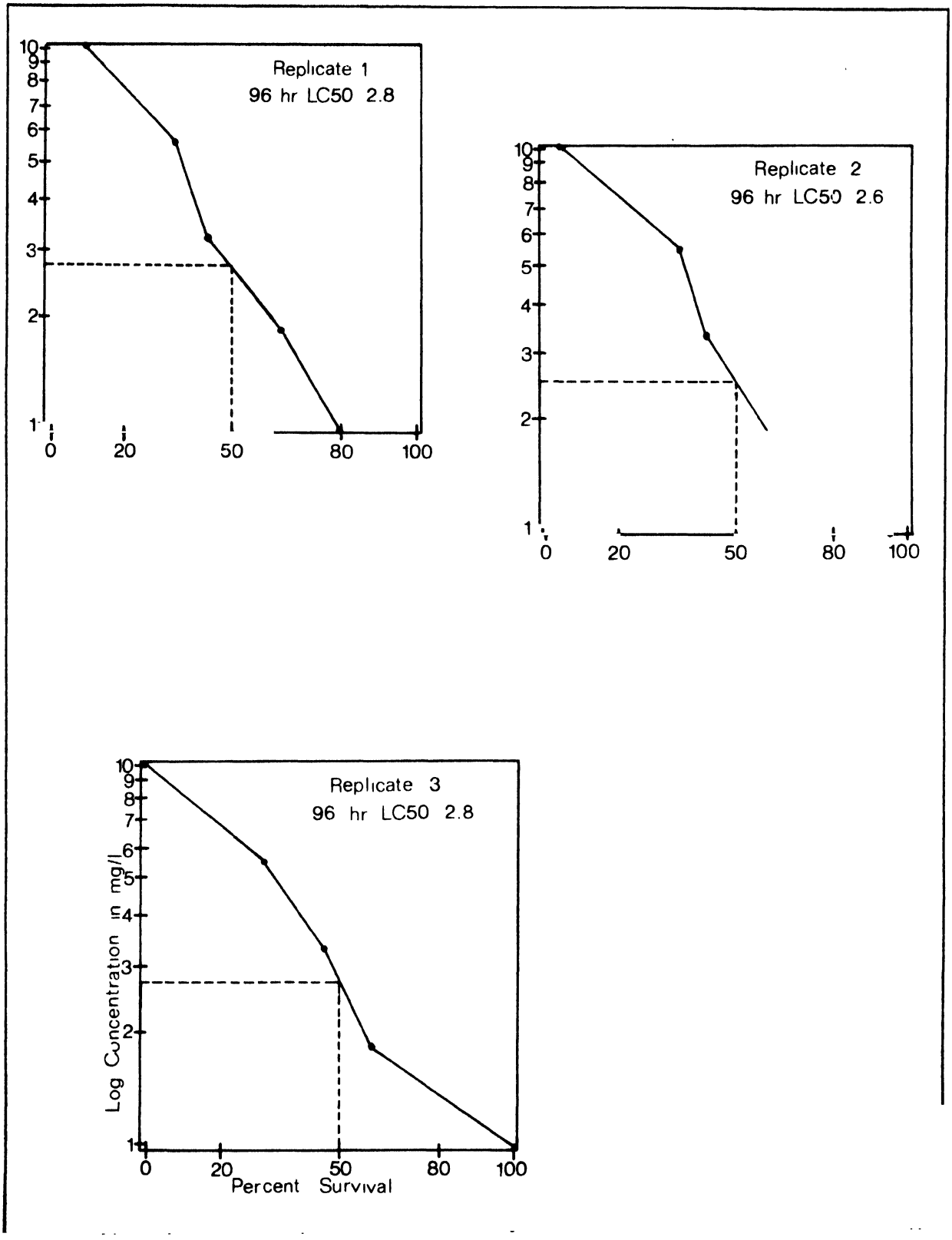

Figure 2. Calculations of the 96 hour LC50 by the logarithmic methods for 3 replicates (Table III).

Table IV

The percent mortality for replicates 1-3;
data taken from Table III

Concentration in mg/l	Number of Test Animals	Percent Mortality Replicate 1	Replicate 2	Replicate 3
Control	20	0	0	0
1.0	20	20	5	0
1.8	20	35	40	40
3.2	20	55	60	55
5.6	20	65	65	70
10.0	20	90	95	100

Table V

The observed and expected mortality values
(Data from Table III and Figure 4)

Concentration	No. Animals Tested	Observed % that Died(O)	Expected % that Died(E)	O-E	Contribution to $(Chi)^2$
Replicate 1					
Control	20	0	0	0	0
1.0	20	20	18	2	0.0028
1.8	20	35	35	0	0
3.2	20	55	54	1	0
5.6	20	65	72	7	0.025
10.0	20	90	86	4	0.013
					0.0408
Replicate 2					
Control	20	0	0	0	0
1.0	20	5	26	21	0.135
1.8	20	40	40	0	0
3.2	20	60	56	4	0.006
5.6	20	65	70	5	0.011
10.0	20	95	83	12	0.115
					0.267
Replicate 3					
Control	20	0	0	0	0
1.0	20	0	0	0	0
1.8	20	40	40	0	0
3.2	20	55	55	0	0
5.6	20	70	70	0	0
10.0	20	100	83	17	0.20
					0.20

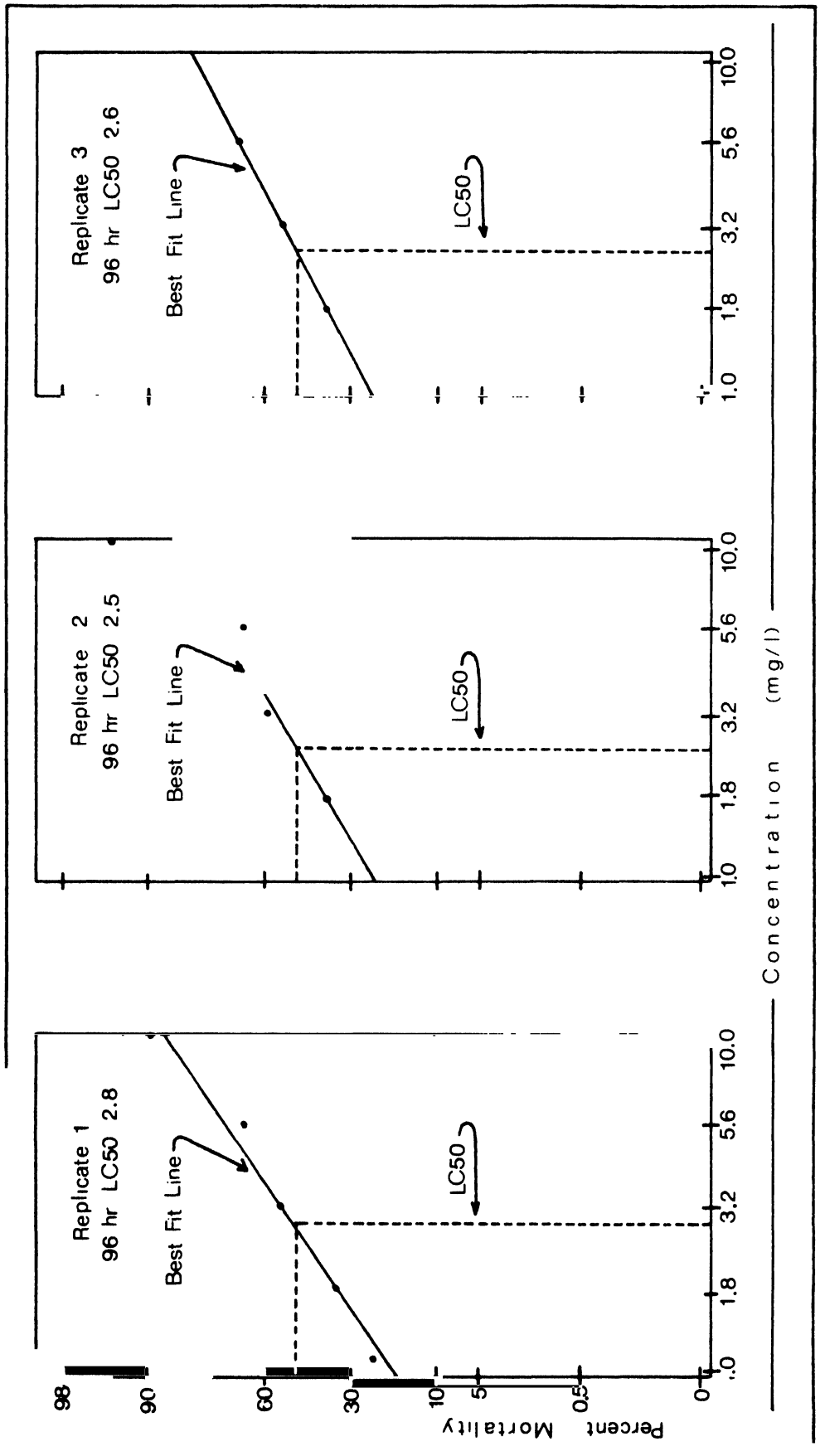

Figure 3. Calculations of the 96 hour LC50 by the probit method for 3 replicates (Table III).

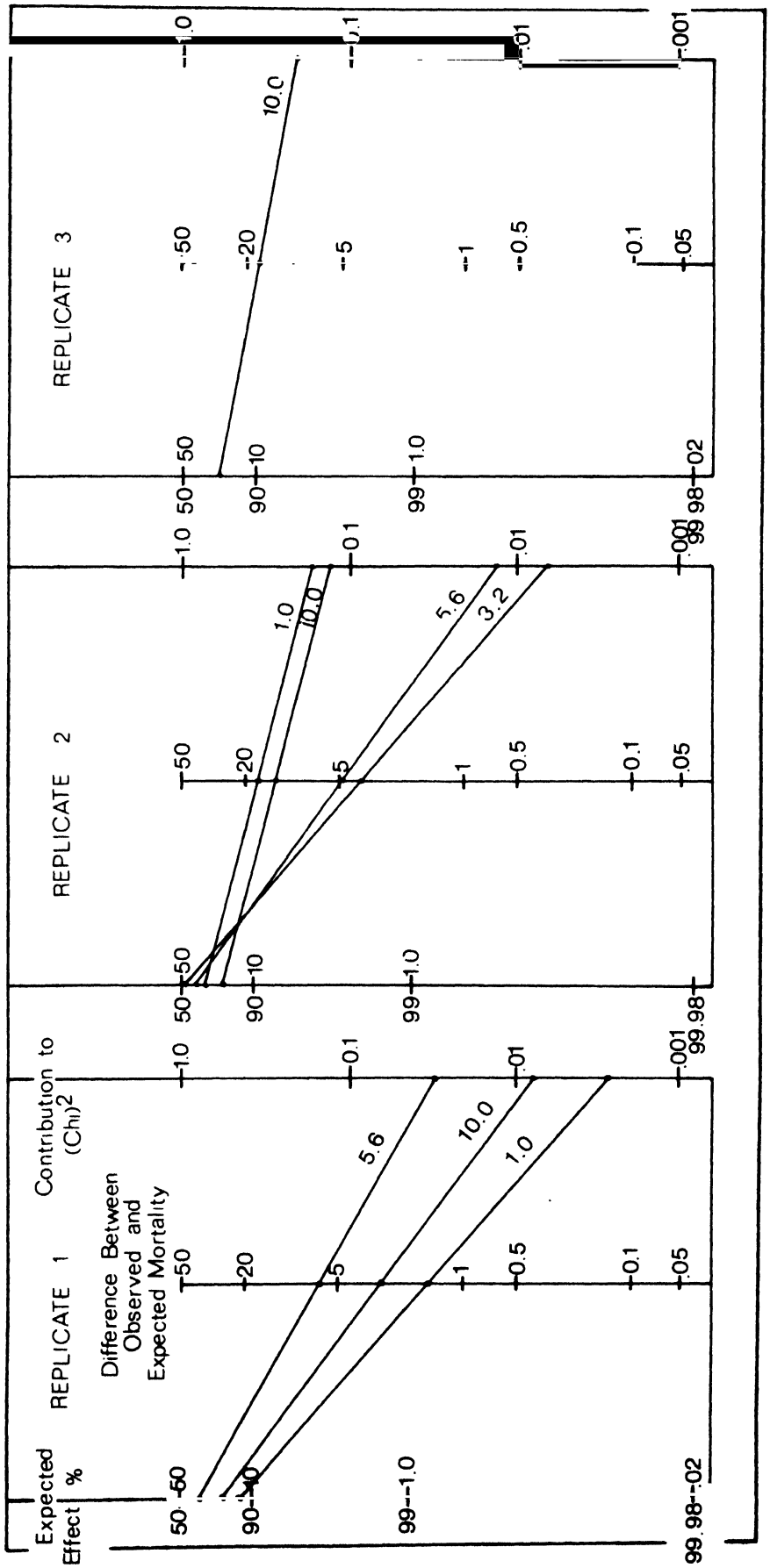

Figure 4. Comparison of the observed and expected mortality for the 3 replicates to determine the validity of the best fit lines in Figure 3.

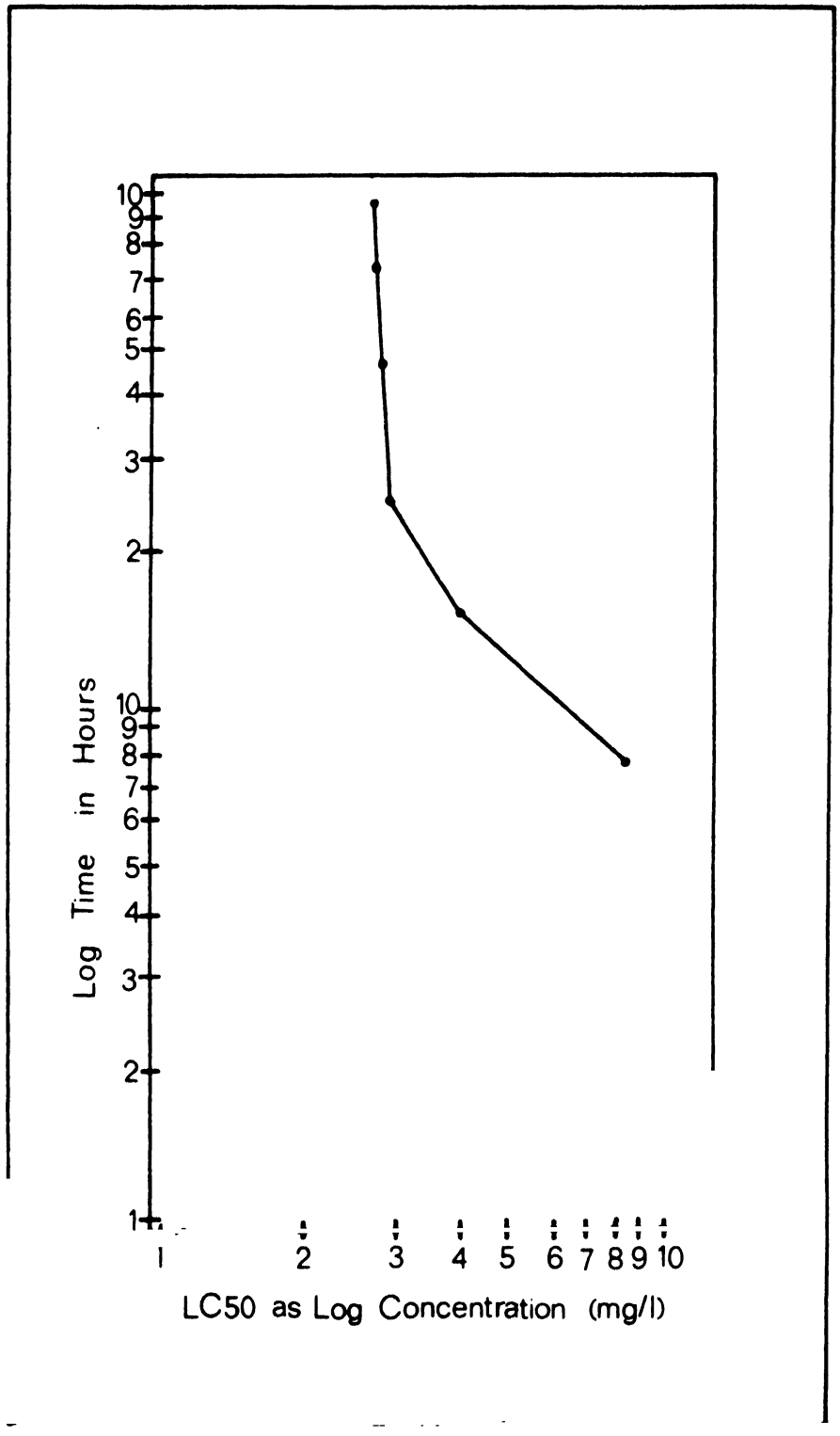

Figure 5. Toxicity curve based on the data in Table VIII.

Inspect the plot visually to see if the line does represent the best fit. If the line does appear to be the best fit, determine the expected effect from the graph for each concentration and compare this with the actual data. The expected mortality and the difference between observed and expected mortality are plotted on a nomograph (Figure 4). A line is drawn connecting these two points and extended to cross the $(Chi)^2$ axis. The $(Chi)^2$ is read from the intersection point of the line with this axis and recorded (Table V). The observed and expected mortality are compared in Table V along with the difference between the two and the individual test concentrations contribution to $(Chi)^2$. The values for each concentration of each replicate are plotted and the $(Chi)^2$ recorded. Note that it is unnecessary to determine $(Chi)^2$ whenever the observed and expected mortality values are identical (see especially Table V, replicate 3). The figures for the contribution to $(Chi)^2$ are added for each replicate (Table V). This total will be used to determine whether or not the lines drawn in Figure 3 represent the best fit (see below).

To determine whether or not the lines in Figure 3 represent the best fit, multiply the total contributions to $(Chi)^2$ by the number of organisms per concentration (20 in this example) and divide by the number of concentrations (6 in this example). (Example - replicate 1: 0.0408 x 20 = 0.8169 divided by the number of concentrations 0.8160 : 6=0.1360). This figure (0.1360) represents the $(Chi)^2$ of the line. It is necessary to determine what the number of degrees of freedom are before the $(Chi)^2$ line can be compared to the $(Chi)^2$ values. The number of degrees of freedom is calculated from the number of concentrations of toxicants used in the experiment. The control is not included. In this example there were five test concentrations and the degree of freedom is two less than this number. Therefore, in this example there are three degrees of freedom (5-2=3). Refer to Table VI and compare the $(Chi)^2$ line (in this example 0.1360) to the $(Chi)^2$ value for three degrees of freedom which is equal to 7.82. Since the $(Chi)^2$ line is less than the $(Chi)^2$ value, the line drawn in Figure 3 is valid and the calculated LC50 is also valid. If, on the other hand, the $(Chi)^2$ line was greater than the $(Chi)^2$ value (for example, 10.3 instead of 0.1360), the line was not a best fit. A new line should be drawn and the same process repeated to check for validity. If, upon doing this, the $(Chi)^2$ line is still larger than the $(Chi)^2$ value, the results of the experiment are not statistically valid and should be discarded and the experiment repeated.

Table VI

Values of $(Chi)^2$ for p = 0.05

Degrees of Freedom	$(Chi)^2$
1	3.84
2	5.99
3	7.82
4	9.49
5	11.1
6	12.6
7	14.1
8	15.5
9	16.9
10	18.3

In the probit method of LC50 calculation, the concentrations at the 16, 50 and 84 percent effects (LC16, LC50, and LC84) are taken from the graph (Fig. 3). For replicate 1, these values are 0.92, 2.8, and 8.8 for LC16, 50, 84, respectively. The slope function (S) is calculated by the formula:

$$S = \frac{\frac{LC84}{LC50} + \frac{LC50}{LC16}}{2}$$

Replicate 1 therefore is:

$$S = \frac{\frac{8.8}{2.8} + \frac{2.8}{0.92}}{2}$$

$$S = 3.09$$

Replicate 2:

$$S = \frac{\frac{10.5}{2.5} + \frac{2.5}{0.6}}{2}$$

$$S = 4.18$$

Replicate 3:

$$S = \frac{\frac{10.0}{2.6} + \frac{2.6}{0.6}}{2}$$

$$S = 4.09$$

Note: In this example it was necessary to project the line beyond the graph to determine both the LC16 and LC84.

The 95% confidence limits are an additional statistical treatment of the data; it defines the limits of the calculated LC50 taking into account the variability of the system. If the 95% confidence limits are narrow, then results of the experiment are considered good. If, on the other hand, the 95% confidence limits are considerable, then the results are not as valid and it might be desirable to repeat the experiment. The 95% confidence limits are useful in comparing the results of one replicate to another. If the confidence limits fall within one another (i.e., 2.2 to 3.9 and 1.9 and 3.7), then the results of these two replicates are the same and are statistically insignificant. On the other hand, if the confidence limits do not overlap (i.e. 2.2 to 3.9 and 0.5 to 1.7), then the differences are statistically significant.

The 95% confidence limits are calculated using the total number of organisms (N) tested at those exposure concentrations whose expected results were between 16 and 84%. From replicate 1 four concentrations (1.0, 1.8, 3.2 and 5.6) had animals that died between the 16 and 84% (Table IV). Therefore, N = 80 in replicate 1. The confidence limits are calculated using the formula:

$$f_{LC50} = S^{\frac{2.77}{\sqrt{N}}}$$

where f equals the confidence limits, S equals the slope function (calculated previously), 2.77 is a constant, and N equals the number of specimens. For replicate 1, this formula becomes

$$f_{LC50} = 3.09^{\frac{2.77}{\sqrt{80}}}$$

$$f_{LC50} = 1.42$$

This result can be determined from a hand calculator which has the capabilities of figuring square root and logarithms (base 10). If a hand calculator is not available it can be determined by the use of logarithmic and square root tables. With the confidence limit f_{LC50} calculated, the upper and lower 95% confidence limits are figures as:

$$\text{Upper limit} = LC50 \times f_{LC50}$$

$$\text{Lower limit} = \frac{LC50}{f_{LC50}}$$

In replicate 1, the 95% confidence limits are:

$$\text{Upper limit} = 2.8 \times 1.42$$

$$= 3.98$$

$$\text{Lower limit} = \frac{2.8}{1.42}$$

$$= 1.97$$

Replicate 2 - 95% confidence limits

$$f_{LC50} = S^{\sqrt{\frac{2.77}{60}}}$$

$$= 4.10^{0.3576}$$

$$= 1.67$$

$$\text{Upper limit} = 2.5 \times 1.67$$

$$= 4.18$$

$$\text{Lower limit} = \frac{2.5}{1.67}$$

$$= 1.50$$

Replicate 3 - 95% confidence limits

$$f_{LC50} = S^{\sqrt{\frac{2.77}{60}}}$$

$$= 4.09^{0.3576}$$

$$= 1.65$$

$$\text{Upper limit} = 2.6 \times 1.65$$

$$= 4.30$$

$$\text{Lower limit} = \frac{2.6}{1.67}$$

$$= 1.56$$

3.1.4 Comparison of the three methods of calculating the 96 hour LC50.

Three methods were presented of calculating the 96 hour LC50 using the results given in Table III; these were: arithmetic (3.1.1), logarithmic (3.1.2) and probit (3.1.3). The 96 hour LC50s for each replicate and each method used in calculating this value are presented in Table VII. The calculated LC50s by the three methods are essentially identical in this example. The arithmetic method is the easiest and

quickest way of determining the 96 hour LC50 and this method will probably be satisfactory in most cases. The probit method requires the greatest amount of time to calculate the 96 hour LC50. The advantage of this particular method is it provides a more reliable statistical treatment of the data. Comparisons between replicates and experiments can be made easily and with statistical validity; that is, by determining whether or not the confidence limits fall within or outside one another. In the example calculated with the data in Table III and given in Table VII, all three confidence limits fall within one another and therefore the three 96 hour LC50s are statistically identical.

Table VII

Comparisons of the 96 hour LC50 calculated by three different methods based on the data given in Table III

Replicate	Arithmetic Method	Logarithmic Method	Probit Method
1	2.8	2.8	2.8 (1.97-3.98)*
2	2.6	2.4	2.5 (1.50-4.18)*
3	2.7	2.6	2.6 (1.65-4.30)*
Average	2.7	2.6	2.6

*95% confidence limits

Table VIII

The effect of a toxicant on an animal as a function of time

Concentration	Time in hours								
(mg/l)	1	2	4	8	16	24	48	72	96
Control	20	20	20	20	20	20	20	20	20
1.0	20	20	20	20	19	19	18	18	16
1.8	20	20	20	19	18	18	16	14	13
3.2	20	19	18	14	11	9	9	9	9
5.6	29	28	26	12	8	7	7	7	7
10.0	19	16	13	9	8	5	3	3	2
LC50	-	-	-	8.5	4.0	3.0	2.9	2.8	2.8

3.1.5 Toxicity Curves

The publication of toxicity curves in research reports or scientific journals is a rare occurrence; however, they are a useful method of analysis to indicate the validity

of the 96 hour LC50 determination. The LC50 is calculated for each observational period over the 96 hour test period as indicated in Table VIII. The LC50 values are then plotted on two cycle log-log paper with the LC50's on the ordinate axis and time in hours along the abscissa (Fig. 5). If the curve becomes asymptotic, as shown in Figure 5, then the 96 hour LC50 is a reasonably accurate figure and the test considered valid. If, on the other hand, the curve does not approach a vertical asymptote, the test cannot be considered valid because the test concentrations were too high or the test organisms used were not in good health, had not been kept in the laboratory for a sufficient holding period, or may not be a good test organism for bioassays. Toxicity curves are useful in preliminary range finding tests as an aid in planning toxicant concentrations for the final test.

3.1.6 Statistical comparison between the effects of the different toxicant concentrations.

Two types of statistical analysis will be calculated to examine the effects of different toxicant concentrations used in the bioassay. First, a single factor analysis of variance (ANOVA) will determine if there is a statistical difference in the number of survivors between concentrations (groups). This will be done by either accepting or rejecting the null hypothesis (H_o) which, in this case, is that there is no statistical difference between the means of the replicates of each of the groups(u) ($H_o = u_1 = u_2 - u_3 = u_4 = u_5 = u_6$). If a statistical difference is found, a multiple range test (Student Newman-Keuls) will be used. The Student Newman-Keuls test will determine which groups (concentrations) have significantly different results from the others.

Table IX

The effect of a toxicant on an animal
(Rearrangement of the data from Table III)

Number of Survivors

Replicate Number	Concentration in mg/l					
	Control	1.0	1.8	3.2	5.6	10.0
1	20	16	13	9	7	2
2	20	19	12	8	7	1
3	20	20	12	9	6	0

3.1.6.1 ANOVA

For convenience in the ANOVA the data from Table III are rearranged accordingly in Table IX.

The total number of data points in the experiment is represented by the formula:

$$N = \sum_{i=1}^{k} n_i$$

where the summation (Σ) of the numbers of experimental groups (k), and n_i represent the number of replicates. Therefore, in this example k is equal to 6 and n_i equals 3; the summation becomes 18 making N = 18. The data within each group (concentration) are totaled and the means calculated. The total number of survivors per group is squared and divided by n_i (=3 in this example). These calculations are presented in Table X.

Table X

The size of the sample, the number of survivors, the mean, and calculations for data given in Table III and IX.

Factor	Concentration					
	Control	1.0	1.8	3.2	5.6	1.0
Sample size	3	3	3	3	3	3
No. of Survivors	60	55	37	26	20	3
Mean	20	18.3	12.3	8.7	6.7	1.0
*	1200	1008.3	456.3	225.3	133.3	3.0

*The square of the summation of the number of survivors divided by the sample size.

The results of the six summations (line 4 of Table X) are totaled (SS_G=3026.2). The number of survivors are totaled which equals 201 (Table IX). The number of survivors in each of the 18 data points is squared and totaled (SS_T = 3039). A correction factor (C) is calculated by squaring the number of survivors (201) which equals 40401 divided by N(=18). The correction factor (C) is then equal to 2244.5.

The next series of calculations involves subtracting the correction factor from the total sum of the squares (SS_T) as:

$$3039 - 2244.5 = 794.5$$

Then the correction factor is subtracted from the group sum of squares (SS_G) as:

$$3026.2 - 2244.5 = 781.7$$

Then the corrected group sum of squares is subtracted from the corrected total sum of squares as:

$$794.5 - 781.7 = 12.8$$

The degrees of freedom for the total are figured as N-1 or 18-1=17. The degrees of freedom for the group are figured as K-1 or 6-1=5. The error in the degrees of freedom are calculated as N-K or 18-6=12 in this case. These calculations are summarized in Table XI.

Table XI

Summary of the analyses of variance

Source of Variations	Sums of Squares(SS)	Degrees of Freedom(DF)	Mean squares $\left(\frac{SS}{DF}\right)$
Total	794.5	17	-
Groups	781.7	5	156.3
Error	12.8	12	1.1

The results in Table XI are then subjected to the F test to see if the null hypotheses is accepted or rejected. F is calculated as:

$$F = \frac{\text{Group mean squares}}{\text{Error mean squares}}$$

$$F = \frac{156.3}{1.1}$$

$$F = 142.1$$

Consult Table XII to determine whether or not F = 142.1 is significant (=null hypotheses rejected or not significant (=null hypothesis accepted) at the 0.05 level. Table XII can only be used if there are six test concentrations (DF=5). It will be necessary to consult a statistical book such as Zar (1974) if DF equals some other number than five. Since the error degree of freedom is equal to 12 and 0.05 is the most frequently used test for significance, find the F number in Table XII. It is equal to 3.11 in this example. Since the calculated F (F=142.1) is larger than the 0.05 F value (3.11), the null hypotheses is rejected. With the rejection of the null hypothesis, this indicates that the data obtained from the different concentrations are significantly different, and it is necessary to conduct the Student Newman-Keuls test.

3.1.6.2 Student Newman-Keuls test

Some of the same calculations used in the ANOVA test will be used in the Student Newman-Keuls test. The means are ranked numerically from 1 to 6 with the lowest mean ranked 1 and the highest ranked 6 to eliminate minus numbers. The means (from Table X) are ranked in the same order as:

Rank of Sample	1	2	3	4	5	6
Rank of means	1.0	6.7	8.7	12.3	18.3	20.0

The standard error is calculated for these data by the formula:

$$S.E. = \sqrt{\frac{MS}{n}}$$

Where MS is the error mean square (=1.1 from Table XI) and n is the size of the sample (=3 in this example for the number of replicates).

Therefore

$$S.E. = \sqrt{\frac{1.1}{3}}$$

$$S.E. = 0.61$$

Table XII

Critical Values of the F Distribution
Numerator DF = 5

(Modified from Zar, 1974, reproduced with kind permission of Prentice-Hall, Inc., Englewood Cliffs, N.J., © 1974.)

Denominator DF	0.10	0.05	0.01	0.005	0.001
1	57.2	230	5760	23100	576000
2	9.29	19.3	99.3	199	999
3	5.31	9.01	28.2	45.5	135
4	4.05	6.26	15.5	22.5	51.7
5	3.45	5.05	11.0	14.9	29.8
6	3.11	4.39	8.75	11.5	20.8
7	2.88	3.97	7.46	9.52	16.2
8	2.73	3.69	6.63	8.30	13.5
9	2.61	3.48	6.06	7.47	11.7
10	2.52	3.33	5.64	6.87	10.5
11	2.45	3.20	5.32	6.42	9.58
12	2.39	3.11	5.06	6.07	8.89
13	2.35	3.03	4.86	5.79	8.35
14	2.31	2.96	4.69	5.56	7.92
15	2.27	2.90	4.56	5.37	7.57
16	2.24	2.85	4.44	5.21	7.27
17	2.22	2.81	4.34	5.07	7.02
18	2.20	2.77	4.25	4.96	6.81
19	2.18	2.74	4.17	4.85	6.62
20	2.16	2.71	4.10	4.76	6.46

In the Student Newman-Keuls test each set of data is compared so that with six samples there are 15 comparisons. The 15 comparisons are made in Table XIII and the necessary calculations are defined in the footnotes to this table. The calculated q value is compared to the $q_{0.05}$ value obtained from Table XIV. It can be seen from the conclusion column in Table XIII that all sets of comparisons are significantly different except 6 vs 5 ($q = 2.79$ and $q_{0.05} = 3.08$, since q is smaller than $q_{0.05}$, these two are not significantly different). Therefore, the results for control and 1.0 mg/l concentration (Table III) are not significantly different but all other sets of data are significantly different. In using Table XIV to determine whether or not two sets of data are significant or not, it is important to remember that this particular table can only be used for tests with six concentrations or less.

The following references are useful for providing additional information on the statistical analysis of bioassay data: Zar (1974), Hubert (1980) and Sokal and Rohlf (1981).

Table XIII

Comparison of Samples
Student Newman-Keuls Test (at $P \leq 0.05$)

Comparison B vs A*	Diffrence ($X_B - X_A$)	S.E.	q**	p***	q0.05,12,p	Conclusion
6 vs 1	20-1=19	0.61	31.15	6	4.75	Different
6 vs 2	20-6.7=13.3	0.61	21.8	5	4.51	"
6 vs 3	20-8.7=11.3	0.61	18.52	4	4.2	"
6 vs 4	20-12.3=7.7	0.61	12.62	3	3.77	"
6 vs 5	20-18.3=1.7	0.61	2.79	2	3.08	Not Different
5 vs 1	18.3-1=17.3	0.61	28.36	5	4.51	Different
5 vs 2	18.3-6.7=11.6	0.61	19.02	4	4.2	"
5 vs 3	18.3-8.7=9.6	0.61	15.74	3	3.77	"
5 vs 4	18.3-12.3=6	0.61	9.84	2	3.08	"
4 vs 1	12.3-1=11.3	0.61	18.52	4	4.2	"
4 vs 2	12.3-6.7=5.6	0.61	9.18	3	3.77	"
4 vs 3	12.3-8.7=3.6	0.61	5.9	2	3.08	"
3 vs 1	8.7-1=7.7	0.61	12.62	3	3.77	"
3 vs 2	8.7-6.7=2	0.61	3.28	2	3.08	"
2 vs 1	6.7-1=5.7	0.61	9.34	2	3.08	"

*Two sample ranks with B the larger rank number
**q is defined as the difference between the means of B-A ($X_B - X_A$) divided by the standard error (S.E.)
***p is the number of means in the range of means being tested; for example, in testing between 1 and 6 there are 6 means (p=6), between 2 and 3 there are 2 means (p=2).

Table XIV

Critical values of the q distribution (at 0.05)

Error D.F.	p = 2	3	4	5	6
1	17.97	26.98	32.82	37.08	40.41
2	6.085	8.331	9.798	10.88	11.74
3	4.501	5.910	6.825	7.502	8.037
4	3.927	5.040	5.757	6.287	6.707
5	3.635	4.602	5.218	5.673	6.033
6	3.461	4.339	4.896	5.305	5.628
7	3.344	4.165	4.681	5.060	5.359
8	3.261	4.041	4.529	4.886	5.167
9	3.199	3.949	4.415	4.756	5.024
10	3.151	3.877	4.327	4.654	4.912
11	3.113	3.820	4.256	4.574	4.823
12	3.082	3.773	4.199	4.508	4.751
13	3.055	3.735	4.151	4.453	4.690
14	3.033	3.702	4.111	4.407	4.639
15	3.014	3.674	4.076	4.367	4.595

3.2 Data Analysis for Other Types of Bioassays

There are many different types of bioassays besides the 96 hour test as indicated in Section 1.2.6. Data analysis for these different types of tests will vary as to the nature of the data generated; however, statistical treatment consists of determining at what concentration a significant difference occurs. In subsequent sections different statistical tests will be presented with each type of bioassay.

3.2.1 Growth

Most growth tests require periods of time greater than 96 hours. Growth may be determined by changes in length or weight. Whichever parameter used, test organisms must still be growing in order to be valid. A difficulty of using growth as a measure is it requires extensive handling of the test organisms prior to the initiation of the experiment. This source of error can be minimized by including 20 additional organisms prior to random selection. These 20 organisms are not used in the actual bioassay but serve as a baseline for organisms in all test concentrations.

If wet weight is to be measured, similar procedures for removing excess water must be used; this is particularly critical if the test organisms are small. These difficulties can be overcome by using dry weight which would necessitate the use of an additional 20 organisms for weight determination at the beginning of the experiment. An example of the use of body weight as a measure of growth is given in Table XV. Data are analyzed to determine which sets of results differ significantly from the control; the Student Newman-Keuls test (Section 3.1.6.2) is used for this type of analysis.

Table XV

The Effect of a Toxicant on the Body Weight of an Organism

Concentration in mg/l	Dry Body Weight in grams at end of Bioassay			
	Initial wt.	Replicate 1	Replicate 2	Replicate 3
Control	6.9	11.1	11.4	11.2
1.0	6.9	11.2	10.9	9.7
1.8	6.9	7.5	7.9	6.2
3.2	6.9	6.0	6.1	5.4
5.6	6.9	5.1	4.2	3.0
10.0	6.9	3.0	1.3	-

3.2.2 Reproduction

The numbers of eggs laid or offspring produced are convenient methods of measuring the effects of a toxicant on an organism. Reproductive experiments often require long periods of time and are often not feasible because of space and economic demands. Some macroinvertebrates complete their life cycles within a month and produce sufficient number of offspring to permit statistical analysis of the data. An example of the use of the number of offspring produced as a measure of sublethal toxicity is given in Table XVI. The number of offspring are counted in each concentration and the results compared statistically to the control using the non-parametric Mann-Whitney U-test (Mann and Whitney, 1947).

Table XVI

The Effect of a Toxicant on the Reproduction of an Organism

Concentration mg/l	Initial Number of Animals	Number of Specimens at 28 days
Control	40	159
0.01	40	148
0.18	40	137
0.32	40	117*
0.56	40	19*
1.0	40	10*
1.8	40	5*

*Significantly reduced reproduction (probability ≤ 0.05)

3.2.3 Biostimulation

Several parameters are used to measure phytoplankton productivity but the most widely accepted measure is the dry weight in mg/l. A minimum of three replicates should be used per concentration. The 95% confidence interval can be determined. Calculations of the 95% confidence interval, standard deviation, and tests for significance are based on the data given in Table XVII. The results can be analyzed for statistical significance by ANOVA with Student Newman-Keuls test (Section 3.1.6.2).

Table XVII

The effect of a toxicant on phytoplankton (dry weight in mg/kg)

Test Solution	Days 0	3	5	10	14
Control					
1	0.01	0.07	0.10	0.12	0.12
2	0.01	0.08	0.12	0.13	0.14
3	0.01	0.10	0.14	0.15	0.15
1% Toxicant					
1	0.01	0.9	5.4	7.2	7.5
2	0.01	1.3	6.2	8.0	8.1
3	0.01	1.2	5.9	7.5	7.7
5% Toxicant					
1	0.01	0.04	0.08	0.12	0.12
2	0.01	0.05	0.08	0.13	0.11
3	0.01	0.03	0.09	0.12	0.12
10% Toxicant					
1	0.01	0.01	0.05	0.0	0.0
2	0.01	0.0	0.0	0.0	0.0
3	0.01	0.02	0.05	0.0	0.0

3.2.4 Bioaccumulation

An organism will accumulate the toxicant during a bioassay test. In some cases, the organism can metabolize the toxicant and excrete it, but at other times the toxicant will accumulate in tissues. It is generally necessary to conduct a bioassay longer than 96 hours in order to get greater amounts of the toxicant in the body. Toxicant concentrations are measured by atomic absorption spectophotometry or gas chromatography depending upon the toxicant. An example of the type of data that one can expect from such an experiment is given in Table XVIII. The data are analyzed by ANOVA with the Student Newman-Keuls test (Section 3.1.6.2).

Table XVIII

Body tissue concentrations of a heavy metal in a marine organism

Test Solution	Tissue conc. mg/kg (dry wt)	Mean	Standard Deviation
Control			
1	70		
2	61		
3	83		
		71	± 9
0.1 mg/l			
1	458		
2	425		
3	419		
		434	±17
0.56 mg/l			
1	669		
2	682		
3	701		
		684	±13
1.0 mg/l			
1	2189		
2	2247		
3	1922		
		2119	±142

4. BIOASSAY PROCEDURES WITH SELECTED GROUPS OF ORGANISMS

Given below is a detailed procedure for conducting bioassays with eight different types of organisms, namely, phytoplankton, annelids, zooplankton, crustaceans, aquatic insects, molluscs, echinoderm larvae, and fish. Reference is made to earlier sections to reduce redundacy. A hypothetical example of a 96 hour test period is given for each group of organisms which includes the test procedures and data analysis.

4.1 Phytoplankton

4.1.1 Source of material

It would be much more convenient to obtain a pure culture strain from a supplier rather than attempt to establish a culture from field collected specimens. Specific sources of cultures are indicated in Section 2.1.2.1.

4.1.2 Equipment and supplies required

The specific equipment and supplies required to conduct short-term bioassays with phytoplankton are:

1. Centrifuge (1000 x g)
2. Centrifuge tubes with stoppers
3. Pipettes
4. Filtration apparatus
5. 250 or 500 ml Erlenmeyer flasks
6. Membrane filters (0.45 μ pore size)
7. Analytical balance
8. Autoclave or pressure cooker
9. pH meter
10. Light meter
11. Compound microscope
12. Haemocytometer counting chambers and occular micrometer
13. Portable electronic counter (Coulter counter)
14. Fluorometer to measure chlorophyll a
15. Oven (120°C)
16. Constant temperature room, cold baths, or incubators
17. Cool-white fluorescent lighting to provide 4304 lumens
18. HCl
19. N_2CO_3
20. Aluminum foil
21. Crucibles

4.1.3 Stock cultures

The procedures for maintaining laboratory stock cultures for a marine phytoplankton species (Dunaliella tertiolecta) are given in Section 2.1.3.3. Procedures for culturing fresh water phytoplankton and other marine species can be obtained from the supplier.

4.1.4 Test procedures

All glassware should be cleaned thoroughly to prevent contamination. Wash glassware in non-phosphate detergent and rinse several times with tap water. Rinse with a 10% solution of HCl followed by a neutralized solution of Na_2CO_3. Rinse five times each with tap water and then deionized or distilled water. Cleaned glassware is dried at 50°C in an oven and stored in a dust free cabinet or with the tops covered with aluminum foil.

A minimum of two experiments should be undertaken; the initial one should be a range-finding experiment in which successive concentrations are one order of magnitude apart (i.e. 0.1, 1.0, 10, 100). The second test, or definitive test, should use a logarithmic scale as given in section 2.3.1. Three replicates should be used for control and each test concentration. The flasks should not contain more than 20% fluid because of carbon dioxide limitations. Fill the Erlenmeyer flask with appropriate quantity of test solution (50 ml in 250 ml flask or 100 ml in 500 ml flask). Inoculate each test flask with 1000 cells/ml. To prepare average inoculation of phytoplankton of this concentration, transfer organisms into centrifuge tube and centrifuge at 100 x g for five minutes. Decant the supernatant fluid and fill with either distilled water (freshwater species) or artifical sea water (sea water species). Repeat the previous procedure and resuspend the cells to give a concentration of 1000 cells/ml. For example if there were 4×10^5 cells/ml in the centrifuge tube after resuspension, pipette 0.25 ml into test flask (in case of 500 ml flasks, to be filled with 100 ml test solution).

Place the flasks under a continuous cool-white fluorescent lighting fixture. Temperature conditions should be 24±2°C for fresh water phytoplankton such as Selenastrum capricornutum or 18±2°C for marine phytoplankton such as Dunaliella tertiolecta. Allow the experiment to run for 96 hours although some workers use a 5 to 14 day period. The maximum growth occurs prior to day five for most phytoplankton species.

The results can be measured in several different ways: dry weight of the biomass of phytoplankton, counting the number of cells per unit volume, chlorophyll a concentration, spectrophotometric measurement of absorbance, and total cell carbon content. The latter two methods are not as widely used and will not be described herein.

The dry weight of the phytoplankton is measured by passing the test solution through a pre-weighed filter (0.45 μ pore diameter) and rinsing with distilled water. Dry the filter paper containing the phytoplankton biomass in a 60°C oven for at least four hours, then weigh. A second method involves centrifuging the phytoplankton at 100 x g. Decant the supernatant fluid and wash three times with distilled water. Transfer the phytoplankton biomass to a pre-weighted crucible and dry over night in an oven set at 60°C.

Cell counts can either be made with an electronic particle counter such as a Coulter counter or by direct cell counts. If an electronic particle counter is available, follow the directions for counting phytoplankton as outlined in the manual. In order to make direct cell counts, place a known volume on a haemocytometer, place under a compound microscope, and count the number of cells present. A grid is etched on the haemocytometer so that only a known fraction of the sample is counted. Follow the procedures included with the haemocytometer for counting and conversion factor to obtain the number of cells per ml.

Chlorophyll a is measured by homogenizing the test solution and placing 5 ml in a cuvette for measuring the concentration on a fluorometer. The fluorometer is initially adjusted to zero reading with a cuvette filled with distilled water prior to reading the chlorophyll a concentration.

4.1.5 Results and data analysis

4.1.5.1 Dry weight

Hypothetical results which measured the effect of a toxicant on phytoplankton growth are given in Tables XIX-XX. The analysis of these data for mean, standard deviation, and test for significance was described in Section 3.2.3 on biostimulation.

4.1.5.2 Cell counts

The results of a hypothetical experiment testing the effects of varying concentrations of a toxicant on the population of a phytoplankton species are presented in Table XIX. The data are analyzed according to mean, standard deviation, and ANOVA with Student Newman-Keuls test.

4.1.5.3 Chlorophyll a concentration

The results of the effects of varying concentrations of a toxicant on the chlorophyll a concentration in phytoplankton are given for a hypothetical experiment in Table XX. These data are analyzed according to the mean, standard deviation, and for significance in this table.

Consult "Standard Methods" (APHA/AWWA/WPCF, 1985) or Miller et al. (1978) for additional information concerning bioassays with phytoplankton species.

4.2 Zooplankton

Many different kinds of marine and fresh water zooplankton species have been used in bioassays. Daphnia magna is the most commonly used fresh water species; copepods and mysids are often used as representatives from the marine environment. A list of these species is given in Section 2.1.2.2.

4.2.1 Test organisms

Daphnia magna can be purchased from a biological supply company. This species is one of the most desirable and convenient representatives of fresh water zooplankton. The species of marine copepods and mysids listed under section 2.1.2.2 may not occur at your locality but related species undoubtedly do. Collect and isolate specimens of a marine species and maintain them according to the techniques given in section 2.1.3.4. The procedures described for zooplankton are for the fresh water species Daphnia magna. Modifications from this basic procedure for copepods and mysids will be indicated at the end of the description of techniques with Daphnia in section 4.2.3.

Table XIX

The effect of a toxicant on
the population of phytoplankton

Test Concentration	0 Days	Number of cells/ml at 5 days Replicate 1	Replicate 2	Replicate 3	Mean ± Standard Deviation
Control	1,000	115,000	122,000	113,000	116,700 ±1170
0.1 mg/l	1,000	112,000	117,000	109,000	112,700 ±3300
0.32 mg/l	1,000	98,000	95,000	89,000	94,000 ±3741
0.56 mg/l	1,000	22,000	26,000	18,000	22,000 ±321.0
1.0 mg/l	1,000	0	0	0	0

Table XX

The effect of a toxicant on
the concentration chlorophyll a

Test Concentration	Concentration of Chlorophyll a g/ml at 5 days Replicate 1	Replicate 2	Replicate 3	Mean ± Standard Deviation
Control	0.35	0.48	0.44	0.42 ± 0.05
0.1	0.32	0.43	0.39	0.38 ± 0.04
0.32	0.33	0.29	0.29	0.30 ± 0.02
0.56	0.04	0.07	0.02	0.04 ± 0.02
1.0	0	0	0	0

4.2.2 Equipment and supplies required

In addition to the general laboratory requirements listed in section 2.2.4, the materials necessary are a good source of fresh water (see section 2.1.3.2), dissecting microscope and light, 125 ml Erlenmeyer flasks fitted with stoppers, pipettes, and finger bowls.

4.2.3 Test procedures

Pipette the neonate stage (young Daphnia which have hatched from the mother's brood chamber during the preceding 24 hours) into finger bowls. The neonate stage is of

sufficient size (up to 1.0 mm in length) that their general condition can be observed with an unaided eye; however, you may wish to examine their condition under a dissecting microscope. Change the water in the finger bowls three to five times in order to remove the food and debris. Pipette 10 specimens into 125 ml Erlenmeyer flasks. Use two flasks for each replicate (total 20 specimens), three replicates per concentration, and five concentrations plus control (total number of flasks = 36). Distribute the flasks in a random manner, remove all but 10 ml of water from each flask and introduce the toxicant into each one. Take these 10 ml into account when calculating the test concentrations (see section 5.1.3). Examine each flask daily and note which specimens are immobile. Lack of mobility does not necessarily signify death even after the flask is swirled. Record the immobile as such and at 96 hours place these specimens in clean water and observe for mobility. Some of them will begin swimming and therefore should be counted as survivors. Examine any of the remaining immobile Daphnia under the dissecting microscope to see if there is any movement, especially heart beat. If the heart is functioning, consider these specimens as survivors. If no movement of any type is seen under the dissecting microscope, consider these specimens as dead. As experience is gained with examination of Daphnia, it will be possible to discern heart beat with the unaided eye.

The procedures with copepods are basically identical to those with Daphnia. Transfer a sufficient number of pelagic copepods from the holding aquarium or stock colony. Examine each specimen under the dissecting microscope to make certain that none of them are females carrying eggs. This is of importance because of their short generation time. If not careful, it is possible to have more specimens at the end of the experiment than at the beginning. Do not use females carrying eggs. Set up the bioassay as with Daphnia. Examine each flask and note the number of copepods which have ceased swimming and have fallen to the bottom of the flask. At 96 hours count the number of swimming copepods and examine each non-swimming specimen under the dissecting microscope for indication of survival. If the specimen shows movement in any of its appendages or contractions of the digestive tract, consider it a survivor.

The procedures with mysids are similar to those of the other groups of zooplankton species. The exception is that finger bowls (110 x 80 mm) or similar-type dishes are used for experimental containers. Remove mysids from the stock culture and examine each individual under the microscope for females carrying eggs in their marsupia. Return these gravid females specimens back to the stock colony; do not use these specimens in the bioassay experiments. Place 10 specimens in each container with two dishes per concentration and use three replicates per experiment. The test containers should be randomly placed and the toxicant added. Examine each container daily and record the number of deaths. A specimen which is neither swimming nor moving should be gently touched with a rounded probe. If it does not move, the specimen is considered dead. If, however, there is movement of the appendages or contractions in the digestive tract, the mysid should be considered as alive. Record the number of living and dead specimens for each concentration at 96 hours.

4.2.4 Results and data analysis

The data given in either Tables III or IV can be used as an example of the results that one can expect from a bioassay using zooplankton species as test organisms. Determine the 96 hour LC50 for each of the three replicates by plotting the data as shown in either Figure 1 or 2. Calculate the mean and standard deviation for the three replicates.

Consult "Standard Methods" (APHA/AWWA/WPCF, 1985), Adema (1978), Nebeker (1982) or Nimmo et al. (1979) for additional information concerning conducting bioassays with zooplanktonic species.

4.3 Annelids

Bioassays with annelids are conducted with marine polychaetes and oligochaetes and fresh water oligochaetes. The basic techniques of conducting bioassays with this group are the same regardless of the type of water or group of annelid. The procedures outlined are for a marine polychaete and any deviation from this basic pattern for fresh water oligochaetes is indicated.

4.3.1 Test organisms

The principle species of annelids which have been used in bioassays are listed under Section 2.1.2.3 including collecting techniques. The maintenance and feeding procedures are given in Section 2.1.3.5. Selection of species to use in bioassays depends upon which are available in large numbers, in a convenient size, and will survive under laboratory conditons. Since a minimum of 360 specimens are required to conduct a bioassay with annelids (5 concentrations + 1 control x 20 specimens per concentration x 3 replicates = 360), it will be necessary to collect as many as 500 specimens to insure an adequate number. Specimens should not exceed 6.0 cm in length because larger worms crawl out of Petri dishes and die of dehydration. Before final selection of an annelid test species is made, a preliminary experiment should be conducted for 96 hours with 20 specimens without adding a toxicant, in other words, the "control" phase of a final experiment. If the survival rate is less than 90%, repeat this preliminary experiment until a 90-100% survival value is obtained. If after 2-3 attempts the survival rate is less than 90%, discontinue using this species and try a second one using the same procedures. Do not proceed with the actual bioassay experiment until satisfactory results are obtained with the "control" phase of the test.

4.3.2 Equipment and supplies required

The specific equipment and supplies needed (in addition to the general laboratory supplies listed under Section 2.2.4) to conduct annelid bioassays are a good source of sea water (or artificial sea salts) and/or fresh water as indicated in Section 2.1.3.2, dissecting microscope and light, glass or plastic Petri dishes (preferred size 25 x 100 mm), fine brushes, white enamel pans, and 500 ml Erlenmeyer flasks (if used for test containers instead of Petri dishes).

4.3.3 Test procedures

Remove the worms from the stock supply and place them into Petri dishes. Several worms can be placed in a single Petri dish for a short period of time. Examine each specimen under the dissecting microscope and discard any injured ones or females with developing ova. Place each selected specimen in a separate Petri dish containinng 25 ml of sea water (if 500 ml Erlenmeyer flasks are used, add 100 ml of sea water). Use a minimum of five concentrations plus control with 20 specimens per concentration and three replicates. Select the specimens for the different concentrations by random placement according to the method outlined in Section 2.4.3. Examine and record the condition of each specimen each day. It may be necessary to examine small worms under the dissecting microscope. Record deaths and any indications of stress which are useful indicators of impending death. If the worm should fragment, determine the condition of the anterior end. If it is living, then consider the specimens as living but under stress. Death can be defined as the lack of body movement, lack of movement in the dorsal blood vessel, or fragmentation into several pieces (however, determine the state of the piece containing the anterior end).

Some oligochaetes may require sediment to survive. Do not use sediment unless it is essential for the survival of the species since many toxicants may become attached to the sediment particles. Determine if sediment is required in the initial "control" experiment. If specimens die within the four days, add sediment according to the procedures given in Section 2.1.3.5.

4.3.4 Results and data analysis

The data given in Tables III and IV can be used as an example of the results that one can expect from a bioassay using annelids as the test organism. Determine the 96 hour LC50 for each of the replicates by the arithmetic (3.1.1), logarithmic (3.1.2) or probit (3.1.3) methods. Calculate the mean and standard deviation for the three replicate LC50's.

Consult "Standard Methods" (APHA/AWWA/WPCF), 1980, Chapman et al. (1982), or Reish (1980) for additional information concerning conducting bioassays with annelid species.

4.4 Crustaceans

Many different groups of crustaceans have been used in bioassays in addition to the cladocerans, copepods, and mysids covered in Section 4.2. These species include marine barnacles, isopods, amphipods, and decapods and fresh water mysids, amphipods, and decapods.

4.4.1 Test organisms

Bioassays with barnacles utilize the cypris larval stage and not the adult. Collect and isolate the larvae as specified in Section 2.1.2.4. Bioassay procedures are identical to those described for copepods in Section 4.2.3. The test procedures with fresh water mysids are identical to those described for marine mysids in Section 4.2.3. The test procedures for marine isopods and amphipods as well as fresh water amphipods are identical; marine amphipods will be used as an example for these types of crustaceans. Larval stages of marine and fresh water decapods can be taken from ovigerous females as indicated in Section 2.1.2.4 and bioassays conducted according to the techniques outlines for zooplankton in Section 4.2.3. Bioassays with decapods are identical with the exception of the size of the container; marine shrimp will be used as an example of a decapod.

4.4.2 Equipment and supplies required

General laboratory items are listed in Section 2.2.4. Additional supplies required for crustacean bioassays include: a good supply of fresh water and/or sea water, dissecting microscope and light, pipettes, Petri dishes, and various sizes of finger bowls or similar type of containers for decapod bioassays.

4.4.3 Test procedures

Remove the amphipods from the holding aquarium and examine each specimen under the dissecting microscope for females carrying ova in their marsupia. If a female is carrying ova, return her to the holding aquarium; do not use her in a bioassay experiment. Place two specimens in a Petri dish (100 x 25 mm); it may be necessary to add an inert object, such as a piece of aquarium tubing, for a substrate. Use 20 specimens per concentration with five test concentrations plus control and three replicates. Distribute the test organisms in a random manner as described in Section 2.4.3. Examine each specimen daily for 4 days (96 hours) and record the number of deaths in each concentration. A stressed or dead amphipod will be lying on its side on the bottom of the dish. Examine under the dissecting microscope to determine whether or not the specimen is alive. It may be necessary to gently touch the specimen with a rounded probe to see if any of the appendages move. Consider the specimen as dead if no movement is noted.

Shrimp and other decapods must be isolated because of their cannibalistic tendencies. Ovigerous females should not be used in a 96 hour bioassay. Females carrying eggs can be recognized with the unaided eye. Place shrimp in individual 400 ml jars (85 x 95 mm). Distribute the jars with test organisms in a random manner as indicated in Section 2.4.3. Use 250 ml of test solution in each jar. The size of the test container may vary according to the size of the decapod. The relationship of the weight of the organims to the amount of fluid present should not exceed 1.0 g/l. In the case of the above example, the weight of the shrimp should not exceed 0.25 g wet weight. If the weight exceeds 0.25 g, in this example, use a larger sized test container. Stressed or dead shrimp will lie on the bottom of the test container. Generally, you can determine whether there is any movement in appendages, digestive tract, or heart with the unaided eye. However, you may wish to verify the condition of the animal by observing it under the dissecting microscope.

4.4.4 Results and data analysis

The data given in either Tables III or IV can be used as example of the type of data obtained with amphipods or shrimps. Determine the 96 hour LC50 for each of the

three replicates by plotting the data as shown in either Figure 1 or 2. Calculate the mean and standard deviation for the three replicates.

Consult "Standard Methods" (APHA/AWWA/WPCF, 1985), Lee et al. (1977) and Nimmo et al. (1977) for additional information concerning bioassays with crustaceans.

4.5 Aquatic Insects

Bioassays are conducted with four different groups of aquatic insects: diptera, mayflies, stoneflies, and caddisflies. It may not be possible to conduct static bioassays with some species collected from fast flowing streams. These species will have a low survival percentage during the laboratory holding period. Such species should be avoided unless a flow-through bioassay system is utilized. Species collected from slower moving streams, rivers or lakes will be easier specimens with which to work.

4.5.1 Test organisms

The principle species of aquatic insects which have been utilized as bioassays are listed under Section 2.1.2.5 which includes the collection techniques. The maintenance and feeding procedures are given in Section 2.1.3.7. Selection of bioassay species depends upon what is available in sufficient numbers. A minimum of 360 specimens are required to conduct an experiment consisting of 20 specimens per concentration, 3 replicates and 5 concentrations plus control. Larval stages of insects should be of similar size and, preferably, in the same intermolt stage (which may be difficult to determine for some species). Determination of the number of living and dead insects may create some difficulty since some specimens may molt during the test period. The cast skins superficially resemble a dead specimen. However, the absence of internal organs will be readily apparent upon examination under a dissecting microscope. If the species has not been used previously in bioassays, conduct preliminary tests without a toxicant to determine the suitability of the species as a laboratory test organism. The survival rate should be at least 90% in this preliminary experiment.

4.5.2 Equipment and supplies required

Most of the equipment and supplies required have been specified under Section 2.2.4. A good supply of fresh water, free from chlorine, is needed as described in Section 2.1.3.2. The size of the glass test container can vary from about 8 to 20 litres. Larger sized aquaria are required for carnivorous species. A fine mesh screen should be placed over each aquaria to prevent possible emerging adults from flying away. Sieves with 0.25, 0.5, and 1.25 mm mesh size will be needed for removing test organisms and preparing the sediment for the bioassay.

4.5.3 Test procedures

Place a 4-5 cm layer of fine sediment in the glass containers. This sediment should be similar to what is present in the natural habitat where the specimens were collected. The sediment should either be passed through a sieve with openings less than 0.25 mm to remove any contained animals or dried prior to use. If caddisfly larvae are used, place sand, wood fragments and plant materials retained on a 1.25 mm mesh sieve as well as some small rocks in the container. Twenty specimens can be placed in each aquarium giving a total of 3 aquaria per concentration or a total of 18 for a single bioassay experiment. Follow the random placement procedures outlined in Section 2.4.3. Examine each aquarium daily for dead specimens. Insect larvae usually emerge from the sediment prior to death, whereas, healthy specimens remain within the sediment. Remove those specimens which are on the surface of the sediment and appear dead. Examine these specimens under the dissecting microscope for any movement of the appendages. Return specimen to the aquarium if movement was observed. At 96 hours, remove all insects from the surface of the sediment and determine their condition. Since these animals burrow into the sediment, disturb the surface sufficiently to destroy the former burrows. After the sediment has settled, count the number of new burrows. This will give an indication of the number of living specimens present. If the number of burrows and the number of dead specimens counted total 20, then the number of burrows can be taken as the number of survivors. However, if the number totals less than 20, remove the sediment at the end of the 96 hour test period from each aquarium and wash it through a

0.5 mm mesh sieve in order to retain the living insects on the sieve. Count the number of living insects and add these to the total number of dead specimens observed during the experiment. If the total is greater than 20, it is possible that a molt was counted as a dead specimen. If the total is less than 20, it is possible that a dead specimen had decomposed and the parts passed through the sieve.

4.5.4 Results and data analysis

The data given in Tables III and IV can be used as an example of the type of results that one can expect from a 96 hour bioassay experiment using insect larvae as test organisms. Determine the 96 hour LC50 for each of the three replicates by the arithmetic (3.1.1), logarithmic (3.1.2) or probit method (3.1.3). Calculate the mean and standard deviation for the three replicate LC50's.

Consult "Standard Methods" (APHA/AWWA/WPCF, 1985) Anderson (1980) and Lamb and Bailey (1981) for additional information concerning bioassays with insects.

4.6 Molluscs

The majority of the bioassays conducted with molluscs have utilized pelecypods, especially mussels, oysters, scallops and clams. Aquatic gastropods have been utilized to a limited extent, but chitons, scaphopods and cephalopods have not been used in routine laboratory testing. The techniques for conducting the bioassay are similar for most species.

4.6.1 Test organisms

The principle species of pelecypods which have been used in the past are indicated in Section 2.1.2.6 which includes the methods of collecting the organisms. Maintenance procedures are given in Section 2.1.3.8. Oysters and mussels are generally available in large numbers and should not present any problems in obtaining sufficient numbers for testing. All test organisms should be of a similar size and the size specified. Smaller specimens are preferred because there is less chance of spawning during the bioassay. Animals should be cleaned of all attached fouling organisms and allowed to acclimate in aquaria for at least 7 days prior to use in testing. Aquaria can be used as test containers since these organisms are not cannibalistic. Twenty specimens can be placed in an aquarium measuring 30 x 60 cm with a water depth of about 10 cm. Aeration should not be necessary for a 96 hour test period; however, if specimens die during a preliminary test, it may be necessary to reduce the number of test organisms per aquarium without reducing the amount of water present. Specimens frequently spawn when they are brought into the laboratory. Generally, most of the specimens will have spawned during the acclimation period. If, however, spawning does occur during the test period, the experiment should be discontinued because test organisms may die as a result of the death and decomposition of the gametes. The five test concentrations and control should consist of 20 animals per aquarium with three replicates making a total of 18 aquaria per test.

Healthy pelecypods generally have their shells slightly apart. Their shells are closed tightly when disturbed or under stress. The shells of a dying or dead pelecypod are usually open and will not close when touched. Pelecypods are not necessarily the best test organism for the 96 hour test period because of their ability to keep their shells tightly closed during periods of stress. At least one preliminary test should be conducted before the final test is run to gain familarity in recognizing and interpreting death in pelecypods.

4.6.2 Equipment and supplies required.

The supplies required to conduct pelecypod bioassays in addition to those listed under Section 2.2.4 are 30 x 60 cm aquaria and brushes for cleaning the fouling organisms from surface of the shells. The fresh water and seawater requirements are specified under Section 2.1.3.2.

4.6.3 Test procedures

Remove pelecypods from the holding tanks and make certain the shells are free of fouling organisms. Place the specimens in rows in an enamel pan. This will facilitate random placement of the test organisms in the 18 aquaria. The amount of sea water and toxicant used will depend upon the size of the aquaria. All aquaria should be of the same size. Examine each specimen daily and record deaths. All dead specimens should be removed to prevent fouling of the water. Death in pelecypods is defined in Section 4.6.1.

4.6.4 Results and data analysis

The data given in Tables III and IV can be used as an example of the type of results one can expect from a bioassay using pelecypods as test organims. Calculate the 96 hour LC50 for each of the replicates by the arithmetic (3.1.1), logarithmic (3.1.2) or probit (3.1.3) method. The mean and standard deviation should be determined for the three replicate LC50's.

As noted in Section 4.6.1, pelecypods have the ability to close their shells tightly which presumably keeps (or reduces) the toxicant from coming in contact with the soft parts within the shells. It is possible that all specimens may survive the 96 hour testing period in the highest test concentration. Shell deposition in oysters (Butler, 1966) and the number of byssal threads produced by Mytilus edulis which occur during the course of a 96 hour experiment (Carr and Reish, 1978) have been used as a measure of sublethal effects of a toxicant.

Consult "Standard Methods" (APHA/AWWA/WPCF, 1985), Butler (1966), Calabrese et al. (1973, Carr and Reish (1978) and Davis and Calabrese (1964) for additional information concerning conducting bioassays with pelecypods.

4.7 Echinoderm Larvae

Most bioassays with sea urchins and sand dollars utilize the gametic and larval stages. Due to the high sensitivity to contaminants, alterations in the fertilization and development of these easily obtainable stages have proven to be effective indicators of seawater toxicity. Gametes can be obtained from the mature echinoids according to the techniques outlined in the section 4.7.3. Bioassay procedures with sea urchins and sand dollars are similar.

4.7.1 Source of material

Sea urchins are collected in the field as indicated in Section 2.1.2.7. They should be packed in seawater-moist (not soaking-wet) towels to prevent desiccation, transported to the laboratory as soon as possible, and induced to spawn immediately or held in seawater aquaria at a temperature similar to that of the water at the collection site.

4.7.2 Equipment and supplies required

General laboratory items are listed in Section 2.2.4. Additional supplies required for echinoid bioassays include: a supply of good sea water, compound microscope, dissecting microscope and light, syringe and needle, 0.5 molar potassium chloride (KCl), sterile gauze, adjustable automatic pipettes (1-5 ml and 5-10 ml), stirrers, Sedgewick-Rafter counting chamber, 5 percent borax-buffered formaldehyde, perforated plunger, polyester/nylon screen (44μ mesh size), 15 ml polyethylene culture tubes, centrifuge, acidified ethanol, 95% ethanol (1 part 25% of HCL and 3 parts 95% ethanol), spectrophotometer cuvettes, and spectrophotometer.

4.7.3 Test procedures

An 850 ml aliquot of test solution is poured into a 1-litre beaker and a 50 ml aliquot is poured into a polypropylene cup (cup capacity approximately 100 ml). This process is repeated four more times as there will be 5 replicates per test solution. The beakers are used for egg and larval exposure, while the cups are for sperm exposure.

In addition to the test solution beakers and cups (5 x number of test solutions), there are at least five "control" replicates containing clean sea water. The temperature of the test solutions is maintained at the water temperature of the adult collection site (see below).

The adult sea urchins or sand dollars are induced to spawn by injecting 0.5 ml of 0.5 M potassium chloride into the coelom. The injected sea urchins are placed on seawater-wet paper towels, oral side downwards, and observed for spawning. The urchins release gametes from the aboral surface; sperm are nearly white and the eggs yellow to orange-brown.

Spawning, female urchins are inverted, aboral side downwards, onto beakers filled to the top with sea water. The diameter of the beaker should be smaller than the diameter of the echinoid specimen. This allows the echinoid to remain "perched" on the beakers with the gonadopores immersed in water. Eggs are only collected during the initial 15 minutes of spawning to minimize the use of immature eggs that might be shed with prolonged spawning. Eggs collected from about six females are combined, passed through sterile gauze to remove spines and debris, and allowed to settle for about 10 minutes. The eggs are "washed" by: decanting off the seawater above the settled eggs, refilling the beaker with clean seawater, stirring the eggs, and allowing the eggs to resettle. This "washing" procedure is repeated once. After completion of the "washes", the eggs and sea water are mixed with a perforated plunger to achieve a uniform egg density and volumetrically sampled (1 ml is removed) with an automatic pipette. The eggs in the sample are immediately counted using the Sedgewick-Rafter counting chamber. When the density of the eggs is known, approximately 30,000 eggs are pipetted into each beaker of test sample by using a perforated plunger to attain a uniform egg density and an adjustable-automatic pipette to deliver the exact volume of egg solution to each test solution. The eggs are held in the test solutions for exactly 30 minutes prior to the addition of the sperm.

Spawning, male echinoids are kept out of sea water with their oral side on seawater-wet towels. This allows collection of sperm under "dry" conditions. In this manner the sperm are not activated by the seawater. Sperm of several males are removed with a pasteur pipette to a test tube and kept cool. Twenty drops of sperm are added to 100 ml of sea water in a 200 ml beaker and thoroughly stirred. This addition of sperm to sea water "activates" the sperm.

Fifteen minutes after the eggs have been added to the test samples, 1 ml of sperm solution is added to each of the test exposure cups (not to the beakers). At this time, the eggs and sperm are simultaneously, yet separately, being exposed to the test solutions.

Fifteen minutes after the sperm are added to the test exposure cups, the exposure cup (sperm) solutions are added to their respective exposure beakers (eggs) to allow fertilization.

Fifteen minutes after the addition of the sperm to the eggs, the eggs in each beaker are mixed to achieve a uniform egg density and a 10 ml sample is taken (by auto-pipette) for subsequent examination of the percentage of fertilized eggs. Each sample is placed in a vial, preserved with 5% borax-buffered formaldehyde, and saved for future microscopic examination.

The remaining eggs are stirred at 60 rpm, aerated, and kept at the seawater temperature of the adult sampling site. The result is a nearly homogeneous egg solution with very slight egg sedimentation. After 48 h, two sets of samples are taken. One sample, to examine larval development, is pipetted in the same manner as the previous sample taken after 15 minutes. The other sample is taken for analysis of echinochrome pigment synthesis and involves a sampling technique described below.

The formaldehyde-preserved egg (15 min) and larval (48 h) samples are microscopically (100x) examined and counted on a Sedgewick-Rafter counting chamber. For the 15 minute samples, "fertilization" of the eggs is defined by the presence of an obvious fertilization membrane around each egg; for the 48 hour samples, "normal" development is defined by the presence of the archenteron at the gastrula stage. The

numbers and percentages of both fertilization and normal development are recorded for each test solution.

For the analysis of echinochrome synthesis, embryos and larvae contained in 800 ml of each test sample are extracted for echinochrome. Embryos are removed from each test solution with a polyester/nylon screen (mesh size 44 µ) and transferred to a 15 ml polyethylene culture tube containing sea water. After packing these echinoid embryos and larvae by centrifugation (1000 x g for 20 min), the sea water above the pellet is removed by pipette. 10 ml of 95% ethanol are added to each pellet. The pellet and solution are vigorously stirred and centrifuged (1000 x g for 15 minutes) to remove interfering substances (e.g. chlorophyll). Following removal of the ethanol solution above the pellet, 3 ml of acidified ethanol (1 part 25% HCl and 3 parts 95% ethanol) are added to each pellet. Extraction of the echinochrome into the acidified ethanol is rapid once the pellet is disrupted with a stirring rod. Extracts are centrifuged (1000 x g for 15 min) to remove any remaining embryo debris. The echinochrome content of each sample is determined by measuring the absorbance of the orange-colored supernatant at 475 nm with a spectrophotometer. The concentration of echinochrome in each sample is proportional to the absorbance. Amounts of echinochrome are calculated relative to the "controls". The echinochrome extracts can be stored at 0°C for up to 72 hours before absorbance must be measured. Storage at this temperature for this period of time causes less than 5% loss of pigment absorbance.

4.7.4 Results and data analysis

For the 48 hour procedure, mortality should not be greater than 15 percent and preferably less than 10 percent in the controls. Regard any mortality more than 15 percent as unsatisfactory and repeat the test under more suitable conditions. Make corrections for mortality or reduced proportion of normal in controls by Abbott's formula (Tattersfield and Morris, 1924) $P = \frac{P^*-C}{}$ where P is the corrected proportion and P* is the observed proportion in the test solution. "C" is the mean proportion in the control test solutions. Test sample toxicities causing 50% response levels (EC50) are then estimated for the fertilization, development, and echinochrome data using the probit method (Section 3.1.3). When compared with the controls, significant reductions in fertilization (15 minutes), or larval development (48 hour) in the test solutions can be attributed to the toxicity of the test solutions.

It is not usually necessary to examine fertilization, development, and echinochrome synthesis in each bioassay. The bioassayist must consider the time involved with the examination of each parameter and accordingly, make his or her decision of what to examine. Consult Bay *et al.* (1983), Kobayashi (1972), or Oshida *et al.* (1981) for further information on this technique.

4.8 Fish

This group of animals is the most difficult one with which to conduct bioassays because of their size and high activity. One should not attempt to conduct bioassays with fish, without previous experience with invertebrates as test organisms. Fresh water fish should be used for the initial bioassays especially if specimens are readily available from a hatchery. Important experience will be gained by working with fresh water species before conducting tests with species of marine fish. The basic techniques of conducting bioassays with this group are the same regardless of the type of water.

4.8.1 Test organisms

The principle species of marine and fresh water species of fish which have been used in bioassays are listed under Section 2.1.2.8. Maintenance and feeding procedures are given in Section 2.1.3.10. While it would be desirable to use 20 specimens per replicate, 10 specimens per replicate with three replicates are frequently used because of space limitations or cost. A total of 180 specimens will be required to conduct one test (5 concentrations and 1 control x 3 replicates = 180 specimens). Test organisms should be of the same approximate size. A desirable size is an 8 cm long fish weighing 5 grams (Sprague, 1973). The largest one should not exceed the shortest one by more than a factor of 1.5. However, 10 litres of test solution are required per gram of

fish. Placing 10 test specimens, weighing 5 grams each, in a single test container would require a 500 litre aquarium. Because of the size of the container required under these conditions, it would be prudent to use smaller size fish or larval stages. It is imperative to conduct preliminary tests to determine whether a particular fish species is a suitable test organism before setting up a final fish bioassay experiment.

4.8.2 Equipment and supplies

Specific requirements in addition to those specified in Section 2.2.4 include appropriate size glass aquaria, nets, aeration, pH meter and the necessary equipment and supplies to measure dissolved oxygen concentrations either chemically or electronically. Small nets are needed to transfer fish from the holding tank to the test container; a separate net is required for each test concentration. The pH should be monitored in every fresh water bioassay; it is not as important in marine systems because of the buffering capacity of sea water. Aeration should be provided to each aquarium if required to maintain a minimum dissolved oxygen level of 5.0 mg/l.

4.8.3 Test procedures

Examine the fish in holding tanks to make certain that none are injured, diseased or parasitized. If six tanks are being used, the first six fish to be caught should be randomly placed in the six test containers. A simple technique for random placement is to have six cards numbered from 1 to 6; place the cards face down and draw one after the fish has been caught in a net. This procedure reduces the handling of the fish. If more than one aquaria is used per concentration, then the number of numbered cards should correspond accordingly. Aeration should be provided if it was determined to be necessary in the preliminary testing. Temperature conditions should be constant, and all aquaria should be exposed to equal amounts of light. If artifical light is provided, the day-night cycle should conform to the natural cycle at the time of the experiment.

Observations should be made at 0.25, 0.5, 1.0, 2, and 4 hours on the first day. Particular note should be made of any abnormal swimming behavior or deaths. Examine each aquarium daily for 96 hours. Measure pH and dissolved oxygen concentrations in each tank daily. Do not feed the fish one day prior to the test nor during the 96 hours testing period; this will minimize the accumulation of fecal material. Death is defined as a lack of movement by the operculum and/or floating without movement.

4.8.4 Results and data analysis

The results in Tables III or IV can be used as an example of the type of data which this experiment would generate. However, since only 10 rather than 20 specimens are being used per concentration and since there may not be any replicates, it will not be possible to determine the mean and standard deviation for the 96 hour LC50s. The 96 hour LC50 can be calculated by arithmetic (3.1.1), logarithmic (3.1.2) or probit method (3.1.3).

Consult "Standard Methods" (APHA/AWWA/WPCF, 1985), Abram and Sims (1982), Alexander *et al.* (1981), Sprague, (1969, 1970) and Verne *et al.* (1982) for additionnal information on conducting bioassays with fish.

5. BIOASSAY PROCEDURES WITH SELECTED TOXICANTS

Given below is a description of the methods and materials used in setting up a bioassay with five different types of toxicants. Emphasis is placed on the procedures used in making the different concentrations of toxicants. Reference is then made to that section which deals with the specifics, i.e., random placement of specimens, etc.

5.1 Metals

It is convenient whenever possible to use the chloride of a metal, such as $CdCl_2$, as the toxicant since it will have a lesser effect, if any, than other anions.

5.1.1 Equipment and supplies required

1. $CdCl_2$ (anhydrous)
2. Distilled water (deironized, if possible)
3. Balance (accuracy to 10 mg)
4. 1 litre volumetric flask
5. 0.1, 1.0 and 10.0 ml volumetric pipettes
6. 1 litre Erlenmeyer flasks
7. 500 and 1000 ml graduated cylinders
8. Filtered fresh water or sea water

5.1.2 Procedures for mixing solutions

1. In order to obtain stock solution of 1000 mg of Cd/l, 1.0 gram of Cd is added to 1 litre of distilled water. The amount of $CdCl_2$ which contains 1.0 g of Cd ions is determined as follows:

$$\text{grams of } CdCl_2 \text{ containing 1.0 g Cd}: \quad \frac{(\text{molecular weight } CdCl_2)}{\text{molecular weight of Cd}}$$

atomic weight of Cd : 112.41
atomic weight of Cl : 35.45
molecular weight of $CdCl_2$: 183.31

$$\text{grams of } CdCl_2 \text{ containing 1.0 g Cd}: \quad \frac{183.31}{112.41} = 1.631$$

2. Weigh 1.631 g $CdCl_2$ (anhydrous) on a balance with an accuracy of 10 mg.

3. Fill a 1.0 litre volumetric flask with approximately 750 ml of distilled water. Add the 1.631 g of $CdCl_2$ to the flask and add additional distilled water slowly until the 1.0 litre level is reached. This stock solution of 1000 mg Cd/l will be useable for about one week.

5.1.3 Preparation of test solutions

A small quantity of stock solution is pipetted from the stock solution and added to either fresh water or salt water depending upon the test organism used. Use the volumetric type pipette rather than a graduated pipette for greater accuracy. If possible, verify the actual concentration of cadmium in each solution by atomic absorption spectophotometry.

1. Test solutions are prepared according to the following chart:

Test concentration	ml of stock solution	ml water
10.0 mg/l	10.0	990.0
5.6	5.6	994.4
3.2	3.2	996.8
1.8	1.8	998.2
1.0	1.0	999.0
0.56	0.56	999.44
0.32	0.32	999.68
0.18	0.18	999.82
0.1	0.1	999.9

2. If Petri dishes are being used as the test container, measure the appropriate amount of test solution into the petri dish so that it is approximately two-thirds full.

3. Select healthy organisms and randomly place them in the test container according to

the procedures outlined in Section 2.4.3.

4. Make observations during and at the conclusion of the test according to Section 2.4.4.

5. Analyze the data according to the procedures given in Section 3.

5.2 Petrochemicals

Oil varies considerably in its toxicity according to its origin in the case of crude or unrefined oil or the method of refining. Crude oil is less toxic than refined petroleum. Another source of variability in petroleum is the length of time the product has been exposed to air. The highly volatile components evaporate largely within a 24 hour period. In order to minimize these variabilities it is important to state the source of crude oil or the type of refined oil used. Furthermore, in order to standardize the method of preparation of test solutions, a procedure was developed by the American Petroleum Institute (Anderson et al., 1974) to eliminate the lower carbon chain gasses (the voltile component) from the oil and utilize the water soluble fraction of the particular petroleum. Since the volatile fraction evaporates within a 24 hour period following an oil spill, the use of the water soluble fraction in toxicity testing approximates what is actually occurring in nature.

Since there are so many variables involved when conducting bioassays with petroleum products, it is important that the concentration of the water soluble fraction be determined for at least the stock solution. Infrared spectophotometry is used to determine the total hydrocarbon concentration in the water soluble fraction. Gas-liquid chromatographic analysis is needed if the identity and concentration of the individual hydrocarbons are required.

5.2.1 Equipment and supplies required

1. Oil (crude or refined)
2. Sea water or distilled water
3. Magnetic stirrer
4. 100 and 1000 ml graduated cylinders
5. 1 litre Erlenmeyer flasks
6. Glass tubing
7. 0.1, 1.0 and 10.0 ml volumetric pipettes
8. Test containers

5.2.2 Procedures for mixing solutions

1. Place 900 ml of either filtered sea water of distilled water together with 100 ml of oil in a one litre flask. Do not place a stopper on the flask.

2. Put a teflon-covered magnetic bar in the flask and place the flask on a magnetic stirrer. Adjust the speed of the stirrer so that the vortex in the flask does not extend more than 25% of the distance from the top of the fluid to the bottom of the flask. Stir the oil-water mixture for 20 hours.

3. After 20 hours turn off the stirrer and allow the mixture to sit undisturbed for 30 minutes. Two layers of fluid exist with the oil present in the upper layer and the water soluble fraction in the lower level.

4. Siphon the water soluble fraction through glass tubing into a one litre Erlenmeyer flask. Place a stopper on the flask until used in the bioassay.

5. Analyze for hydrocarbon concentration if either infrared spectrophotometer or a gas-liquid chromatographic instrument is available.

5.2.3 Preparation of test solutions

1. Select the type of test container appropriate for the size of the test organism. Consult Section 4 to determine what kind of container is

appropriate for the different types of test organisms.

2. The stock solution is diluted with either distilled water or filtered sea water to obtain the desired concentration. The amount of stock solution required to mix a particular concentration is obtained by multipling the percent concentration by 1000; this figure subtracted from 1000 gives the volume of water required to make one litre of test solution. For example, if a 56% concentration is desired, then 0.56 x 1000 = 560; add 560 ml of stock solution to a one litre flask together with 440 ml of water.

3. Place the appropriate volume of solution into the test container.

4. Select healthy organisms and randomly place them in the test container according to the procedures outlined in Section 2.4.3.

5. Make observations of the test organisms for survival and death during and at the completion of the experiment according to the procedures given in Section 2.4.4.

6. Analyze the data according to the procedures given in Section 3.

5.3 Pesticides

Pesticides can be characterized by their target organism, such as insecticides, herbicides, fungicides, rodenticides, to mention the more common types. Pesticides can also be characterized by their chemical nature, such as chlorinated hydrocarbons, organic phosphates, or carbamates. Chlorinated hydrocarbons include DDT, the best known of all pesticides; lesser known compounds include chlordane, benzene hexachloride, lindane, dieldrin, aldrin, endrin, toxaphene, methoxychlor, and heptachlor. Parathion, malathion, and tetraethyl pyrophosphate are among the better known organic phosphate pesticides. Sevin is the principle compound belonging to the carbamates.

Since most of the pesticid developed in the past few decades are synthetic organic compounds, they may possess long half-lives and therefore will persist in the environment for a long time. Furthermore, pesticides are generally not specific so their effect may cause unintentional damage to the environment. The tendency in recent times is to develop more specific target pesticides which have a very short half life in the environment.

Many pesticides have a low solubity in water and require an organic solvent, such as acetone, in order to get the compound in solution for testing. It is necessary to include a second control with the solvent by itself in each experiment. Since DDT has been the most widely used pesticides, it will be used as an example of this type of toxicant.

5.3.1 Equipment and supplies required

1. DDT (technical grade)
2. Acetone
3. Balance with accuracy to 10 mg
4. 100 ml volumetric flask
5. 50,100, 500 ml graduated cylinders
6. 0.01 to 10 ml volumetric pipettes
7. 1 litre Erlenmeyer flasks
8. Distilled water or filtered sea water
9. Gas chromatograph

5.3.2 Procedures for mixing solutions

1. Weigh out 1.0 g of technical grade DDT on an analytical balance.

2. Place 90 ml of acetone in a 100 ml volumetric flask. Add the 1.0 g of DDT to the flask and slowly add additional amounts of acetone to make a 100 ml stock

solution of DDT. The concentration of DDT is 10,000 mg/l in this example.

3. Mix well by shaking the stoppered flask so that all the DDT is dissolved in the acetone.

4. If possible, verify the concentration of DDT and the congeners DDD and DDE in the stock solution by gas chromatography.

5.3.3 Procedures for conducting bioassays

1. Select the appropriate sized test container according to the species used as outlined in Section 4.

2. Determine the quantity of DDT stock solution required for each solution as indicated below. Pipette the quantity of stock solution required with a volumetric pipette and add to either a measured quantity of fresh water or sea water. The amount of stock solution and water required to mix different concentrations of DDT are:

Test Concentration of DDT	Amount of Stock solution	Amount of Water
10.0	1.0	999
5.6	0.56	999.44
3.2	0.32	999.68
1.8	0.18	999.82
1.0	0.1	999.9
0.56	0.056	999.944
0.32	0.032	999.968
0.18	0.018	999.982
0.1	0.01	999.99

3. Measure out the required volume of test solution and place in the containers.

4. Select healthy organisms and randomly place them in the test container according to the procedures outlined in Section 2.4.3.

5. Observe the test organisms for survival and death during and at the completion of the experiment according to the procedures given in Section 2.4.4.

6. Analyze the data according to the procedures given in Section 3.

5.4 Contaminated Sediments

Periodically it is necessary to dredge navigable estuaries, harbours, rivers, or lakes because of siltation. Disposal of dredged sediments may create problems if the material is contaminated with toxicants. Sediments are disposed on land, other parts of the body of water, or at some distance from the dredged site. If the contaminated sediments are disposed elsewhere such as out at sea, the toxicant present with the sediments may adversely affect the organisms present at the disposal site. Techniques for testing the potential toxicity of contaminated sediments on organisms have been devised by a joint effort of the U.S. Environmental Protection Agency/Army Corps of Engineers (1977). Techniques are described which test the effects of dredge spoils and sediments. Since toxicity in the water column is only temporary but can be long term in the benthos, the methods described herein only address the toxic effect of the sediments which settle on the benthos.

5.4.1 Equipment and supplies required

1. Boat equipped for benthic sampling
2. Benthic sampler (for example a Peterson, van Veen, box corer)
3. Large plastic bags (20-25 litre capacity)
4. 0.5 mm sieve

5. Aquaria (50-60 litre capacity)
6. Fresh or sea water supply

5.4.2 Procedures for collecting contaminated sediments

1. Collect the sediment in the field with a bottom sampler such as a Peterson.

2. Use only the sediment from the center of the sampler to avoid contamination by the sampler itself.

3. Place the sediment in a clean plastic bag. It may be necessary to take additional benthic samples from the same locality in order to obtain a sufficient quantity.

4. If the toxicants are high in organics such as oil, use clean glass containers instead of plastic bags.

5. If available, place the sediment in a refrigerator while on board the boat. The sediment should be transported to the laboratory as quickly as possible.

5.4.3 Procedures for conducting sediment bioassays

1. The sediment is sieved through a 0.5 mm mesh sieve to remove organisms and larger sediment particles. Use as little water as possible.

2. Allow the water and sediments to settle for six hours. Decant or siphon off as much water as possible and mix the sediments thoroughly. The sediments can be stored in the damp condition for up to two weeks at 2-4°C. Neither dry nor freeze the sediments.

3. Place 3 cm of sediment in each aquaria. A typical 55 litre aquarium requires about 5200 ml of sediment to obtain this depth. Slowly add 20 litres of water and allow the sediment a minimum of three hours to settle.

4. Use three aquaria containing contaminated sediment for replicates. A control can consist of either uncontaminated sediments or aquaria lacking sediment.

5. Place a minimum of 20 specimens in each aquarium.

6. It will not be necessary to change the water in each aquarium if the test is conducted for only a 96 hour period, however, since many contaminated sediment bioassays go for 10 to 20 days, it will be necessary to renew some of the water. Remove 50-75% of the water at 48 hour intervals by siphoning off 10-15 litres of water then slowly add the same amount of clean water in the aquarium making certain to disturb the sediments as little as possible.

7. Observe the aquaria each day for dead organisms. Stressed animals generally come to the surface prior to death. Determine whether or not the test organisms lying on the surface of the sediment are living or dead. If the organism does not move, remove it and check closer or examine it under a dissecting microscope. Return the test organism to the aquarium if movement is observed.

8. At the end of the test period, remove the water, then wash the sediments through a 0.5 mm mesh sieve to collect the test organisms. If the number of dead organisms removed during the test period and the number of living organisms retained on the sieve do not total the original number, it is possible that some of the test organisms died and decomposed during the test period. In this instance the number of deaths would be equal to the original number minus the number of living organisms observed at the end of the test period.

9. Data analysis in this example with contaminated sediments are different than for other experiments because only one "concentration" is used. Determine the

percent survival, mean and standard deviation for both the contaminated and clean sediment test series. If the standard deviations do not overlap, then the differences are significant. If, however, they do overlap, then the differences are not significant.

Consult the manual written by the U.S. Environmental Protection Agency/Army Corps of Engineers (1977) for detailed procedures in conducting contaminated sediment bioassays. The papers by Swartz et al., 1979, 1982 give results of sediment bioassays.

5.5 Liquid effluents

In most bioassays the toxicant is weighed out, a stock solution is made, and the varying test concentrations are made. The specific concentrations of each test concentration is known. However, in some instances it is necessary to conduct a bioassay with a liquid effluent in which the composition and concentration of the different chemicals are unknown. Examples of liquid effluent wastes from sewage treatment plants, from industry, or a combination of one or more of either or both of these. This type of test is useful in determining the water quality of a body of water which receives waste discharges from many sources.

5.5.1 Pre-bioassay analysis

Prior to conducting a bioassay with a liquid effluent, try to determine the nature and concentration of the wastes in the discharges. This information will be important in determining what chemical(s) to analyze and what instrument(s) to use. Use both atomic absorption spectophotometry and gas chromatography to make these analyses. It is important to remember that the concentration of a particular chemical in a liquid effluent may vary according to time. Samples should be taken at various time periods to account for this veriability.

5.5.2 Equipment and supplies required

1. Distilled water (deionized, if possible)
2. Sea salts (if marine bioassays are planned)
3. Atomic absorption spectophotometer
4. Gas chromatograph
5. 1 litre volumetric flask
6. 1 litre Erlenmeyer flasks
7. 10 litre flask
8. 500 and 1000 ml graduated cylinder
9. Filtered fresh water or sea water

5.5.3 Procedures for mixing solutions

1. Collect eight 1.0 litre samples of the liquid effluent over a 24 hour period in order to minimize variability in the sample (APHA/AWWA/WPCF, 1985). Mix all samples together in a 10 litre flask. Filter the water if particulates are present. Analyze this sample for metals, petrochemicals and pesticides with atomic absorption spectrophotometry and gas chromatography. Make three analysis with each instrument and determine the mean for each chemical.

5.5.4 Procedures for conducting bioassays

1. If fresh water animals are being used as test organims, make dilutions with deionized water. If seawater animals are being used in the tests, adjust the salinity to 32-35 $^{o}/_{oo}$ S with the addition of sea salts. It will be necessary to include an additional control of the sea salt to account for this additional variable.

2. The amount of stock solution required to make a particular concentration is determined by multyplying the percent concentration by 1000; this figure subtracted from 1000 gives the volume of water required to make one litre of test solution. For example, if a 56% concentration is desired, then 0.56 x

1000 = 560; add 560 ml of effluent to a one litre flask together with 440 ml of water.

3. Place the appropriate volume of solution into the test container.

4. Select healthy organisms and randomly place them in the test container according to the procedures outlined in Section 2.4.3.

5. Make observations of the test organisms for survival and death during and at the completion of the experiment according to the procedures given in Section 2.4.4.

6. Analyze the data according to the procedures given in Section 3.

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